

REVIEW

Sizing up models of heart failure: Proteomics from flies to humans

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Cardiovascular disease is the leading cause of death in the western world. Heart failure is a heterogeneous and complex syndrome, arising from various etiologies, which result in cellular phenotypes that vary from patient to patient. The ability to utilize genetic manipulation and biochemical experimentation in animal models has made them indispensable in the study of this chronic condition. Similarly, proteomics has been helpful for elucidating complicated cellular and molecular phenotypes and has the potential to identify circulating biomarkers and drug targets for therapeutic intervention. In this review, the use of human samples and animal model systems (pig, dog, rat, mouse, zebrafish, and fruit fly) in cardiac research is discussed. Additionally, the protein sequence homology between these species and the extent of conservation at the level of the phospho-proteome in major kinase signaling cascades involved in heart failure are investigated.

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1 Introduction

Cardiovascular disease (CVD) is the leading cause of death in the Western world. Each year, CVD results in approximately 700 000 deaths in the United States, and 1 900 000 deaths in the European Union (EU) [1,2]. The economic burden is enormous, and the annual cost is estimated at \$300 billion in the United States and €196 billion in the EU [1, 2]. Thus, there is a pressing need to improve diagnostic and treatment options. Proteomics can be used to explore the underlying biosigna-

ture during the development and progression of heart failure (HF). This includes characterization of signaling and post-translational modification (PTM) regulation pathways, which may lead to new drug targets for therapeutic intervention. In addition, proteomic approaches can identify and validate new circulating biomarkers to improve disease diagnosis and risk stratification, allowing for earlier and more efficacious patient treatment.

Cardiovascular proteomics began with the seminal work by Dunn's group using 2DE to separate myocardial proteins [3]. At that time, the major challenge was identifying the proteins within each gel spot [3], which was revolutionized with the application of mass spectrometry (MS). Since then, the field of proteomics has matured, allowing for rapid and precise determination of absolute quantity of individual proteins as well as individually modified amino acid residues. This review will concentrate on (i) the application of proteomics to HF, (ii) the use of animal model systems (pig, dog, rat, mouse, zebrafish, and fruit fly) to study this complex condition, (iii) the determination of proteins with the highest

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Abbreviations: **AKAP**, A-kinase anchor proteins; **CVD**, cardiovascular disease; **ECM**, extracellular matrix; **EU**, European Union; **HF**, heart failure; **MI**, myocardial infarction; **MMP**, matrix metalloproteinase; **PDE**, protein phosphodiesterase; **TAC**, transverse aortic constriction; **UAS**, upstream activation sequence; **VAD**, ventricular assist devices

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degree of amino acid sequence homology between these different species, and (iv) the similarity of phospho-proteome and kinase signaling cascades between human and the various model organisms.

2 Human heart failure

HF is a debilitating disorder defined as a deficiency in the capability of the heart to adequately pump blood in response to systemic demands [2]. The initiating and underlying causes of HF are often medical conditions such as hypertension, myocardial infarction, coronary artery disease, ischemia, myocarditis, valvular heart disease, congenital malformations, and commonly, cardiomyopathies [4, 5]. Hypertrophic cardiomyopathy, characterized by a thickened but nondilated left ventricle, has a prevalence of 1:500 [6]. Dilated cardiomyopathy, in which one or both of the ventricles are enlarged with accompanying systolic dysfunction, is less prevalent (1:2500), although it is the most frequent cause of heart transplantation [6]. The least common form is restrictive cardiomyopathy, which results in impaired diastolic filling of the ventricles [6, 7].

The progression toward end-stage HF involves broad cellular changes including reactivation of the fetal gene program (e.g. upregulation of atrial and brain natriuretic peptide, skeletal α -actin, β -myosin heavy chain, and fetal type cardiac ion channels), cardiomyocyte hypertrophy, mitochondrial dysfunction, and alterations in calcium handling and sarcomeric proteins that result in part from the dysregulation of intracellular kinase signaling cascades and other protein PTM pathways [4, 8]. The heterogeneous responses differ from patient to patient and depend on environmental and lifestyle factors, which hamper the development of new interventions. Further complications arise from the plethora of genetic mutations that can lead to inherited diseases that frequently promote HF [9–12].

The availability of both healthy and diseased human cardiac tissue samples presents a significant challenge to the study of HF. To study the progression of cardiac diseases, tissue biopsies or samples obtained during surgery are needed, but availability is limited. Furthermore, complex patient phenotypes due to overlapping pathology (e.g. diabetes or hypertension) frequently reduce experimental reproducibility and restrict tissue utility. End-stage HF samples are most commonly obtained either at the time of device implantation (such as ventricular assist devices, VADs) or during transplantation or other invasive procedures. Control tissue samples often comes from donor hearts that are not suitable for transplantation or that have other underlying issues, making these samples ill representative of a “healthy” individual. Therefore, the use of experimentally malleable animal models and their corresponding primary or derived cell lines is necessary. Lifestyle and environmental factors are controlled in most laboratory animals, and inbreeding restricts genetic variability. The underlying assumption is that the various model systems,

e.g. pig/swine (*Sus scrofa*), dog/canine (*Canis lupus familiaris*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), zebrafish (*Danio rerio*), and fruit fly (*Drosophila melanogaster*), recapitulate aspects (physiological, cellular, and/or molecular) of human (*Homo sapiens*) CVD.

This assumption is predicated on a relatively high degree of protein conservation across the animal model systems and the human cardiac proteome. Figure 1A shows the number of myocardial proteins with $\geq 90\%$ amino acid sequence homology between human and large (pig and dog), medium (rat and mouse), and small (zebrafish and fruit fly) models (based on proteins specifically annotated to be present in human cardiac muscle within the Uniprot database, www.uniprot.org). The vast majority ($>98\%$) of protein sequences within this database are derived from the translation of coding sequences present in publicly accessible nucleic acid databases. Not all proteins are represented in all species, however, and not all entries have been observed at the transcriptional or protein level. Interestingly, the percent overlap between the human proteome and that of large or medium animal models is considerable, but this conservation drops dramatically between the human and small animal model systems in which there are few proteins with a high degree of amino acid homology. Not surprisingly, as the stringency for conservation at the amino acid level is reduced the percent overlap between human and all model systems increases dramatically.

Despite disparate predicted numbers of highly homologous myocardial proteins, each model organism has advantages and disadvantages with respect to their biological complexity, translational effectiveness, throughput, and genetic flexibility. This correlates with differences in the ease of use, cost and resources required to carry out experimental studies, and not surprisingly with the numbers of papers published with a particular model system (Fig. 1B). In Fig. 1C, Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed>) queries were performed to obtain papers published involving the various species surveyed in this paper. The limitations on our numbers may be due to the shortcoming of the database used. However, the search features enabled by Pubmed provides a tool for advanced queries, which allows sorting papers as reviews, clinical, or topic-specific papers. The greatest number of published papers pertaining to myocardial and HF proteomics (clinical and basic research combined) is based on human cardiac tissue (Fig. 1C).

2.1 Swine and canine models of HF

Pigs and dogs possess a high degree of overall conservation of the human cardiac proteome (Fig. 1A), which is highlighted by their physiological and anatomical similarities. While the challenges of working with large animals are great, and genetic manipulation is extremely challenging, they represent the most translational models where therapeutic interventions can be readily carried out. In 1962, one of the first animal models of HF was generated by applying chronic tachycardia

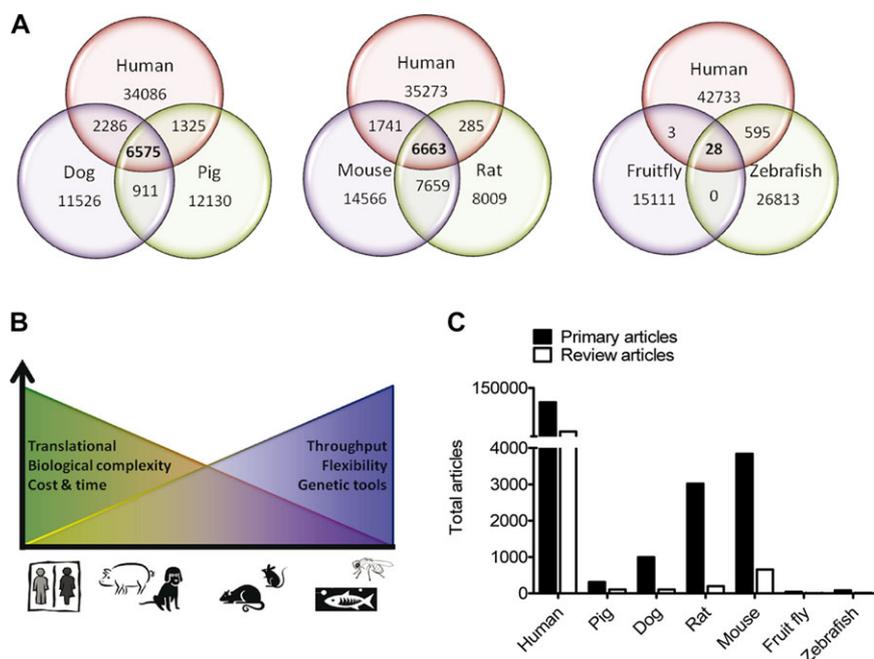


Figure 1. (A) Venn diagrams showing the number of proteins with $\geq 90\%$ amino acid sequence homology between human, dog and pig (left); human, mouse and rat (middle); and human, zebrafish, and fruit fly (right) proteomes. CD-HIT generated protein clusters at $\geq 90\%$ homology were used to identify representative proteins common across species and unique to each species, and used to create the Venn diagrams. (B) Summary of the advantages and disadvantages of the various animal models for HF research. (C) Number of peer-reviewed articles about HF on the publicly available database Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed/>), including reviews and primary articles using human, pig, dog, rat, mouse, zebrafish, or fruit fly models. The bar graph for human includes basic and clinical HF research. Pubmed was queried for all scientific papers published with key word “heart failure” from 1916 to August 2013. A total of 131 643 articles were returned.

(rapid pacing) in a dog using an implantable pacemaker [13]. Due to its size, the dog was well suited for these early studies since technology used in humans could be directly utilized. The tachypacing-induced HF model recapitulates much of the dysfunction seen in humans and is still used in research [14]. This technique has since been employed to generate HF in pigs [15] and sheep [16], as well as to study various pacing-induced conditions, such as cardiac dyssynchrony [17, 18]. Another common approach to generate HF in large animals is via volume overload in which the chordae tendinae is severed to induce mitral regurgitation [19, 20]. These HF models have also proven to be very useful as preclinical support for large clinical trials. For example, validation for angiotensin II receptor blockade in HF was obtained from large animal models [21, 22]. State-of-the-art HF therapeutics, including gene [23, 24] and stem cell-based therapies [25], use large animal models as a translational platform. Because of their size, they can be used to test implantable technologies ultimately intended for humans, such as VADs [26, 27]. However, these surgeries are complex, requiring sophisticated surgical suites and highly trained personnel, which leads to one of the most obvious challenges when exploiting large animals: cost. The procedures, equipment, surgical areas, personnel, housing, feeding, and other animal costs are all significantly greater than those of rodents. However, the dog heart is ~ 1000 times larger than a mouse heart (~ 150 mg [28] vs. ~ 150 g [29]), and many researchers harvest multiple usable samples from a single animal, which helps offset the cost burden.

Another significant drawback when using large animals for basic science research is the lack of genetic tools. Unlike mice and smaller models, there is little ability to control the genome of dogs and pigs, and there are relatively few inbred strains that can be used to reduce variability. In fact, the only

genetic tools available are those available to humans, such as gene therapy.

The use of large animal models of HF predates much of the proteomics field. Compared to the smaller animal models, few cardiac proteomic studies have been conducted in pigs and dogs (Fig. 1C). Nevertheless, the use of 2DE [30, 31], MS [31, 32], and reverse-phase protein microarrays [33] have been used to identify protein changes in HF. Additionally, cutting-edge proteomics techniques such as multiple reaction monitoring (MRM) have been used to determine changes in the phosphorylation state of single troponin I residues in the tachypaced HF dog model [34]. Proteomics in large animals has also been applied to investigate other cardiovascular disorders including ischemia/reperfusion injury [35, 36], myocardial stunning [37], cardiac dyssynchrony [18], and hypoxia/reoxygenation injury [38].

Despite the high degree of amino acid sequence homology, difficulties with large animal myocardial proteomics still subsist. For example, it is our experience that many antibodies that work in humans or rodents are ineffective in dogs or pigs. This most likely results from small differences in the targeted amino acid sequence, which significantly reduce antibody-binding affinity. Most commercially available antibodies are neither designed nor tested with large animal studies in mind. Moreover, protein and peptide identification using MS-based approaches is challenging because of the relatively small-annotated databases (e.g. uniprot) available for large animals compared to human and rodent. The reduced proteome coverage of these species often results in a reduction in the number of proteins resolved as well as the sequence coverage of these proteins, which can have an even larger effect on the number of PTM residues identified.

2.2 Rats and mice, the most commonly used animal models

Rodent (rat and mouse) models are extensively used in CVD research and recapitulate etiologies such as aortic stenosis and hypertrophic, restrictive, and dilated cardiomyopathies [39]. There are more HF publications based on rat and mouse models than that of larger animal systems (Fig. 1C). Of the various rodent models of cardiac dysfunction, it is particularly common to use surgical intervention to generate HF. In mice and rats, the aorta can be constricted using a suture or clip to elicit chronic hypertension-induced HF [40]. In mouse models, the most commonly used technique is transverse aortic constriction (TAC), and in rat models of pressure overload the abdominal aorta is clipped above or below the renal arteries [41, 42]. Another common surgically induced model of HF is performed by either permanent occlusion of the left anterior descending coronary artery or occlusion for a specific time period followed by reperfusion to mimic myocardial infarction (MI) [43]. Following surgery, the heart undergoes distinct anatomical and functional changes due to the hypertrophic growth of the ventricles and subsequent cardiac dilation. Another pathologic insult includes exposure to toxic compounds. Early studies observed that isoproterenol administered to animals produced cardiac hypertrophy, which can decompensate to HF [44]. Doxorubicin is a cardiotoxin that has clinical application as a cancer therapeutic, and exposure in rat and mice results in dose-dependent dilated cardiomyopathy [45, 46]. An additional, distinct model of cardiomyopathy begins with inflammation of the heart (myocarditis) and is produced in rodents by exposure to pathogens including coxsackie-B3 virus, encephalomyocarditis virus, and cardiac myosin peptides [47–50]. In an effort to more fully recapitulate the features of human cardiomyopathy, researchers have also developed models of HF exhibiting comorbidities such as diabetes, which may be induced by giving rodents streptozotocin and hypertension models with ischemia [51, 52].

Genetic models of cardiomyopathy have been and continue to be created by deletion or overexpression of proteins implicated in disease pathways. For example, in mice the cardiac specific overexpression of $G\alpha_q$, a membrane-associated protein that can trigger signaling cascades through activation of phospholipase C, leads to hypertrophic growth and subsequent cardiac dilation and dysfunction [53]. Myocarditis that occurs as a result of a pathologic signaling cascade involving TNF- α may be induced by cardiac-specific overexpression of the protein [54]. Moreover, transgenic mice have been established that model hypertrophic cardiomyopathy due to introduction of an arginine⁴⁰³ to glutamine mutation into the α cardiac myosin heavy chain gene [55], and restrictive cardiomyopathy by expressing mutant cardiac troponin I (arginine¹⁹² to histidine mutation) [56]. Genetic rat models of HF that are frequently used include spontaneously hypertensive rats, spontaneously hypertensive HF prone rats and Dahl salt-sensitive rats, which exhibit hypertension and HF with age or introduction of a high-salt diet, respectively [57–59].

Rodent models of cardiomyopathy are advantageous due to a relatively well-annotated genome and proteome and a reasonable degree of amino acid sequence homology to the human cardiac-specific proteome (Fig. 1A, Supporting Information Fig. 1). Additionally, the rodent heart shares many of the complexities of the human heart including a four-chambered structure, yet there are important physiological differences in the size, heart rate (600/min in mice versus 70/min in human), and in calcium handling. Furthermore, affected human and rodent subjects differ in regards to age (rodents are relatively young), sex (male rodents are commonly used), and comorbid conditions (not commonly modeled in rodents), which may contribute to inconsistencies between human disease and rodent models. Also, the complications of strain differences may confound the exploratory work of different labs. For example, in mouse models of pressure overload, strains respond differently to TAC or MI surgeries as C57BL6 mice exhibit systolic dysfunction and remodeling whereas sv129 mice exhibit preserved systolic function at the same time-point [60, 61]. Thus, interpretations of protective or pathologic effects in the rodent do not always translate to clinical applications. Nonetheless, Drozdov et al. combined different murine cardiac hypertrophy genomic and proteomic datasets to study molecular patterns to distinguish pathological and physiological left ventricle HF [62]. Moreover, proteomic analysis of pressure overload, myocardial infarction, myocarditis, doxorubicin toxicity interventions in addition to the genetically engineered rodents [63–66] can provide interesting new hypotheses and tremendous insight into human HF.

Recently, more awareness has been raised about the extracellular matrix (ECM) proteome. Changes in the protein concentration and modification of a family of ECM proteolytic enzymes, the matrix metalloproteinases (MMPs), have been identified in left ventricle remodeling and can be a contributing factor in the progression to HF [67]. Proteomic analysis of a number of different murine models has enhanced our understanding of ECM proteins and their proteolytic enzymes [68, 69]. Also, new fractionation and extraction methods to improve sample preparation to study MMPs [70] and ECM proteins [71] have emerged allowing broader coverage of this insoluble subproteome of the myocyte and the heart.

2.3 The zebrafish and fruit fly: The smallest animal models used in cardiovascular research

D. melanogaster (fruit fly) has existed as a model organism for genetics research and human biology for over 100 years, beginning with the groundbreaking work of Morgan [72]. *D. rerio* (zebrafish) has been a model for human cardiac disease for a much shorter time period than most other animal models, which helps explain the lower number of total proteomic and cardiac related publications (Fig. 1C). In regard to CVD, both animals have been used as models of dilated and restrictive cardiomyopathies and, to a lesser extent,

hypertrophic cardiomyopathy [73–79]. Even though there is a low number of cardiac-designated proteins with over 90% amino acid homology between flies and humans (Fig. 1A and Supporting Information Fig. 1A), functional conservation is often retained. This conservation can result from individual domains and structural elements of a protein, which are required for a particular function, exhibiting a high degree of amino acid sequence similarity. Other amino acid stretches of the same protein, however, may lack high homology. Since the functionally and structurally important regions may comprise relatively few amino acid residues, much of the remaining protein sequence will be under less evolutionary pressure to retain similarity. Thus, a lack of high degree of homology over the complete sequence may be misleading.

Recently, the physiological assessment of heart function in the adult fly has been greatly advanced using optical coherence tomography and high-speed digital recordings of heart contractions [80, 81], making high throughput measurement of cardiac disease phenotypes more feasible. Zebrafish have been used successfully in drug discovery and chemical screening processes [82, 83]. However, after initial screening using these small animal models, positive hits need to be validated in larger mammalian systems, such as mice or rats and potentially pigs or dogs.

There are many aspects of the fly and zebrafish that make them preferred models for CVD research. One major benefit of *Drosophila* is that their maintenance is more cost effective than that of larger mammalian systems. Moreover, data acquisition is relatively rapid compared to all other animal models. The fruit fly heart is a simple linear cardiac tube consisting of a single layer of contractile myocardial cells and nonmuscular pericardial cells that align along the length of the tube. The morphological differences that exist between the fly and the human cardiovascular system (e.g. single chamber, lack of coronary circulation, etc.) limit the utility of *Drosophila* as an animal model for certain types of human heart disease [84–86]. However, the basic cellular and molecular machinery of the cardiac fibers are highly conserved between *Drosophila* and vertebrates, and, moreover, the embryonic origin and tubular structure are very similar [87–90]. The genome of the fly is much smaller than the human genome, although many disease-related genes found in humans have orthologous genes in the fly. Furthermore, the fly reproduces quickly and has a very short lifespan, which makes genetic crosses and establishment of transgenic lines relatively rapid. The reproductive cycle of a fly is approximately 10–12 days, and adult flies can live up to 2 or 3 months, generating hundreds of genetically identical offspring.

Unlike in *Drosophila*, the zebrafish heart consists of two chambers, one atrium and one ventricle, consisting of both a myocardium and an endocardium [91]. In addition, the zebrafish is a vertebrate and most of its genes have human homologs. The life cycle of the zebrafish, however, is long compared to the fly; it matures within 3 months and has a lifespan up to 5 years.

Both model systems benefit from a robust array of well-developed genetic tools and currently available resources. For example, the UAS-Gal4 system is an inducible transgene expression system in which specific binding of the yeast transcription activator protein (Gal4) to an upstream activation sequence (UAS) activates downstream gene expression [92]. It is commonly used in the fly to study tissue-specific gene expression (e.g. cardiomyocyte-specific expression) [92] and is also a rapidly advancing tool for zebrafish. The Vienna *Drosophila* Research Center (<http://stockcenter.vdrc.at/control/main>) has produced and maintains many publically available UAS-RNAi responder strains that target ~90% of the entire *Drosophila* genome [93]. Flytrap (<http://flytrap.med.yale.edu/index.html>) is an additional, highly valuable resource that distributes publically available transgenic fly lines that harbor a wide range of green fluorescent protein tagged proteins [94, 95].

Both fish and fly model systems possess numerous unique and powerful advantages that have helped advance cardiovascular research and will continue to do so in the future. Despite their small size, which makes tissue procurement challenging, these models are being increasingly utilized for proteomics studies (e.g. MS) [96–99], including the cardiac proteome [89, 100].

2.4 Convergence and divergence across all seven species at different levels of homology

In Supporting Information Fig. 1A we examined the conservation between the proteomes (based on 100, 90, or 80% amino acid sequence homology) of seven species: human, pig, dog, rat, mouse, zebrafish, and fruit fly. Only those proteins observed in all species, and which are also annotated as expressed in the heart based on the human Uniprot database, were included in the comparison. Interestingly, just two proteins are completely homologous (100%) between all seven species: histone 3 and its variant histone 3.3. Histones are proteins closely associated with DNA in the nucleosome core units of chromatin that are essential for life. It is therefore not surprising to have the highest conservation between almost all known species [101]. Histone H3 is one of the five (H1, H2A, H2B, H3, and H4) main histone proteins and has three known sequence variants in mammalian cells, histone H3.1, histone H3.2, and histone H3.3. The variants are highly conserved and differ only by a few amino acids [102].

There are 21 proteins with $\geq 90\%$ and 82 proteins with $\geq 80\%$ sequence homology (Table 1, see online Supporting Information for details). To determine homology, we used CD-HIT Suite [103] to cluster highly similar protein sequences across the different species with varying sequence identity cutoffs. The input used was a combined UniprotKB [104] database (release 2013–08–20) of human, rat, mouse, dog, pig, zebrafish, and fruit fly, and the generated output was a list of clusters with a representative protein for each

Table 1. Proteins common in all 7 species (human, pig, dog, rat, mouse, zebrafish, and fruit fly) with at least $\geq 80\%$ homology

Protein accession	Protein name	Protein accession	Protein name
P62191	26S protease regulatory subunit 4*	P62805	Histone H4*
P35998	26S protease regulatory subunit 7*	Q16576	Histone-binding protein
P62195	26S protease regulatory subunit 8*	Q7L9L4	MOB kinase activator
O00487	26S proteasome regulatory subunit 14	Q15843	NEDD8
P62266	40S ribosomal protein S23	P84074	Calcium-binding protein BDR-2
P46782	40S ribosomal protein S5	P0CG48	Polyubiquitin-C*
P62829	60S ribosomal protein L23	Q6P2Q9	Pre-mRNA-processing-splicing factor 8*
P11021	78 kDa glucose-regulated protein	P41223	Protein BUD31 homolog
P68133	Actin*	P61326	Protein mago nashi homolog
P59998	Actin-related protein 2/3 complex subunit 4	P61619	Protein transport protein Sec61 subunit alpha
P84077	ADP-ribosylation factor 1*	P60059	Protein transport protein Sec61 subunit γ
P18085	ADP-ribosylation factor 4	P61236	Protein yippee-like 3
P62330	ADP-ribosylation factor 6*	P15153	Ras-related C3 botulinum toxin substrate
Q9BXS5	AP-1 complex subunit mu-1	P62820	Ras-related protein Rab-1
Q96CW1	AP-2 complex subunit mu	Q15907	Ras-related protein Rab-11
P53680	AP-2 complex subunit sigma*	P61106	Ras-related protein Rab-14
P25705	ATP synthase subunit alpha, mitochondrial	P61019	Ras-related protein Rab-2
P62158	Calmodulin*	Q9NRW1	Ras-related protein Rab-6
P68400	Casein kinase II subunit alpha	P62834	Ras-related protein Rap-1
P60953	Cell division control protein 42 homolog*	P08134	Rho-related GTP-binding protein
P61201	COP9 signalosome complex subunit 2	P11908	Ribose-phosphate pyrophosphokinase
P24468	COUP transcription factor	P62714	PP2A-beta*
P61962	DDB1- and CUL4-associated factor 7	P62136	Serine/threonine-protein phosphatase PP1
O00148	DEAD box protein 39	P62314	Small nuclear ribonucleoprotein Sm D1
Q9Y295	DRG-1	P83876	Thioredoxin-like protein 4A*
P30876	RNA polymerase II subunit B2	Q15369	Elongin-C
P19388	RNA polymerase I, II, and III subunit ABC1	P55072	Transitional endoplasmic reticulum ATPase
Q96FJ2	Dynein light chain*	Q9Y310	tRNA-splicing ligase RtcB homolog
Q05639	Elongation factor 1-alpha	Q9BQE3	Tubulin alpha chain*
Q9H9T3	Elongator complex protein 3	P68371	Tubulin beta chain*
P38919	Eukaryotic initiation factor 4A-III	P62312	U6 snRNA-associated Sm-like protein LSM6
P62495	Eukaryotic release factor 1	P62979	Ubiquitin-40S ribosomal protein S27a*
Q95166	SABA(A) receptor associated protein	P62987	Ubiquitin-60S ribosomal protein L40*
P62826	GTP-binding nuclear protein Ran	P61077	Ubiquitin-conjugating enzyme E2*
P62879	G protein subunit beta-2	P49459	Ubiquitin-conjugating enzyme E2 A
P11142	Heat shock cognate 71kDa protein	Q9BZL1	Ubiquitin-like protein 5
Q96KK5	Histone H2A	Q9UBQ0	Vesicle protein sorting 29
P0C0S5	Histone H2A.Z	P27449	V-type proton ATPase proteolipid subunit
P62807	Histone H2B	P38606	V-type proton ATPase catalytic subunit A
P84243	Histone H3*	P15313	V-type proton ATPase subunit B

Proteins with $\geq 90\%$ homology have been marked (*).

cluster. CD-HIT was used to identify homologous and unique protein groups between species (Supporting Information Fig. 1A). Table 1 lists the 82 proteins reported to be expressed in the heart (based on Uniprot annotation) and that share a $\geq 80\%$ amino acid homology between all seven species.

There is similar distribution of proteins involved in heart function and disease among these conserved proteins (Supporting Information Fig. 1B shows the functional analysis, generated using IPA (Ingenuity® Systems, www.ingenuity.com) for the proteins listed in Table 1). Perhaps not surprisingly, the top functional categories (assigned by IPA, associated with the 82 proteins) include RNA, PTMs and DNA replication (Supporting Information Fig. 2C). Of these 82 proteins, 11 have been shown to be involved in a

specific cardiovascular function or CVD according to their annotations (Table 1).

2.5 Protein modification: The phosphorylation status in heart failure

Phosphorylation (and other PTMs) modulates cardiac function and is significantly and quantitatively changed with HF [105–107]. For example, disease-dependent phosphorylation of specific cardiac sarcomeric proteins was shown as early as the 1970s to alter heart performance [108–112]. MS has been successfully used to identify the exact location of specific phosphorylation sites, and more recently, the MS-based

MRM (or single reaction monitoring) has been employed to quantitate both unphosphorylated and phosphorylated tryptic peptides to obtain the exact stoichiometry (extent) of protein phosphorylation.

MRM utilizes N^{15} labeled standards consisting of the equivalent synthetic peptides being targeted (note, endogenous peptides are primarily N^{13}), which are spiked into the sample, allowing absolute quantity to be determined. Recently, this tool was used to determine the phosphorylation levels of over 10 modifiable amino acid residues of cardiac troponin I in 30 human heart samples [34]. Phosphorylation of novel C-terminal sites of cardiac troponin I were increased with HF while the classical N-terminal sites (such as those phosphorylated by protein kinase A, G, or C) were decreased. In addition to serine and threonine phosphorylation, a novel tyrosine phosphorylated residue was identified in this study [34], which suggest the existence of a new kinase pathway. Furthermore, using intact protein MS analysis, Ge and colleagues have shown that phosphorylation and other disease induced modifications can occur in vivo [113, 114]). An intriguing, unanswered question is whether the differences in levels of phosphorylation (or other disease-induced PTMs) might serve for HF risk stratification if detected in the blood [34, 115, 116].

In human heart failure, phosphorylation has been the dominant PTM studied, as shown by the high number of published articles (Fig. 2A), compared to those focused on O-GlcNAc or acetylation (as indexed on Pubmed). Our literature search criteria consisted of one of the three PTMs, in combination with the terms “heart failure” and “human.” Ninety-six percent of the results involve phosphorylation, 3.7% acetylation and 0.3% O-GlcNAc. Despite a minimal number of published articles, O-GlcNAc is of interest, as this enzymatically induced PTM also targets S and T residues like phosphorylation, and we are beginning to appreciate its importance in myocardial preconditioning and diabetes. It is important to note that the difference in number of publications available for each PTM does not necessarily indicate its functional importance but merely the ease and available technology to

study certain modifications. This is also reflected by the difference in the extent to which various signaling pathways (TGF β , GSK-3 β , AKT, PKG, PKC, or PKA) have been investigated (Fig. 2B illustrates the number of articles that have been published on HF in human or using a mouse model).

2.6 The GSK-3 β /AKT and PKA pathways are highly conserved

The GSK-3 β /AKT and PKA pathways have been extensively studied in HF (Fig. 2B). AKT has primarily been examined in the mouse, whereas PKA has primarily been investigated in humans. We used STRING (version 9.05, [117]) analysis to create a high confidence co-occurrence diagram to illustrate the presence or absence of 16 proteins within the GSK-3 β /AKT pathway across species (Fig. 3A). This addressed the question of how conserved key proteins are within this pathway across the seven model organisms. The intensity of the color of the red square illustrates the amount of conservation at the amino acid residue level of the homologous protein. Not surprising, GSK-3 β and AKT and some of their targets (e.g. CTNNB1 (catenin β 1), GYS1 (glycogen synthase 1), and SGK3 (serine/threonine protein kinase)) are highly conserved, indicating the functional importance of this pathway for cellular function throughout the animal kingdom. This implies that proteomic changes in GSK-3 β /AKT signaling observed in one species will likely be relevant and translatable to humans. However, other downstream targets like SNAI1 (snail protein), APC (adenomatous polyposis coli), AXIN1 (axin protein 1), PSEN1 (presenilin 1), and FRAT1 (frat1 protein) are conserved between human, dog, rat, and mouse but not swine. This is surprising since it is believed that pig is the most translational animal model for human CVD research, but, as mentioned earlier, only functional and structural motifs need to be highly conserved within a protein in order for it to retain its biological role. We should note that our study is limited by the size and completeness of protein information for each species available in the current Uniprot database. Inadequate protein sequence coverage for any

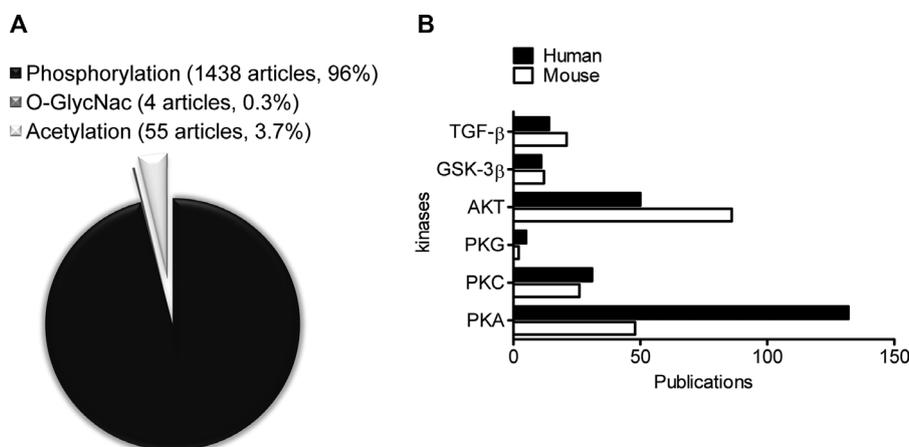


Figure 2. (A) Number of publications on Pubmed involving the PTMs phosphorylation, O-GlcNAc, and acetylation associated with HF. In total, there were 1423, 55, and 4 articles published on phosphorylation, acetylation, and O-GlcNAc modifications respectively. (B) Articles published involving phosphorylation and HF in humans (black bars) or mice (white bars) were sorted by kinase (PKA, PKC, PKG, AKT, GSK-3 β , and TGF- β). Note that if there were articles containing more than one of the kinases searched for, the same article was counted more than once.

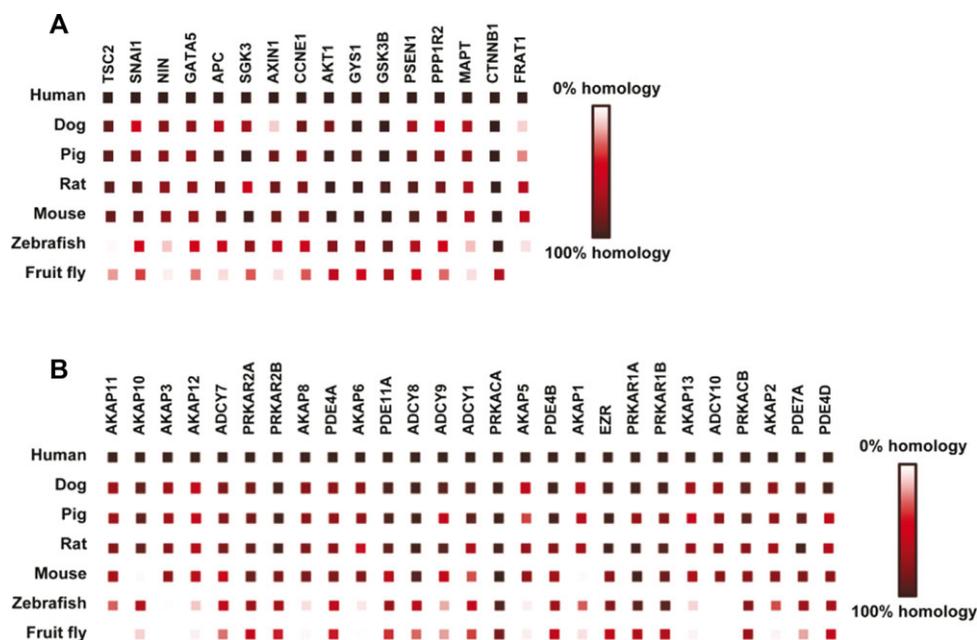


Figure 3. (A) High confidence score based network of the GSK-3 β /AKT and PKA pathways created using STRING [117]. Conservation (amino acid sequence homology) level of GSK-3 β and AKT in the GSK-3 β /AKT pathway across the seven species shows very high homology from human to fruit fly, suggesting their importance in cardiac function. (B) The conservation levels of the individual proteins of the PKA pathway show that all PKA subunits (PRKAR and PRACA) have a high degree of amino acid sequence homology from human to fruit fly.

particular species will result in an underestimation of homology between all of them. The number of proteins listed in Table 1 (proteins with $\geq 80\%$ amino acid sequence homology) will most likely increase as protein annotation improves.

The PKA pathway and 27 associated proteins were analyzed as described above. PKA is composed of two catalytic subunits (PRKACA) and two regulatory subunits (PRKAR) (Fig. 3B). All PKA subunits show a high degree of amino acid sequence homology from human to fruit fly. The A-kinase anchor proteins (AKAP), which bind PKA and target it to different cellular locations, are the least conserved, while the protein phosphodiesterase (PDE) family is as conserved as the protein subunits of PKA. The PDE family controls the quantity of cAMP in a cell, which in turn regulates the activity of PKA. Perhaps these enzymes have a “more essential” role than AKAPs, or there is more redundancy within the AKAP family in which members are able to compensate for each other. Alternatively, the domains for which the individual AKAPs target may exhibit sequence variability. Practically, this information is useful when deciding which species to use when investigating a particular aspect of the PKA pathway. If the goal is to move between model systems or to associate with human cardiac disease, conservation of the pathway is necessary.

2.7 Future directions and conclusion

We believe that with improved technology the identification and characterization of proteins within the heart, as well as inclusion of the complete predicted proteomes of all species within the Uniprot database, will most likely expand the list of proteins with $\geq 80\%$ amino acid sequence homology (Table 1). This will certainly advance the utilization of animal models and translation to human cardiac disease. It is our

hypothesis that these highly conserved proteins are critical and essential for cardiac physiology and life. Deletion of any one of these proteins should be lethal to any cell type and genetic mutations or single-nucleotide polymorphisms are likely to be disease initiating. Future investigations into the regulation of these proteins, e.g. cellular concentration, isoform expression, and PTMs, could provide insight into how cells respond to (disease) stimuli.

Each animal model has advantages and disadvantages, and the best choice for HF research will be dictated by the functional parameters of interest, the need for genetic manipulation, and the overall goal of the study. It is important to remember that the degree of protein conservation within specific pathways or disease processes can influence the extent to which the results are transferable to human pathophysiology. Regardless, quantification of proteins and their modified forms is essential to understand the underlying biology of CVD disease and HF.

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