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Advances in Induced Pluripotent Stem Cells, Genomics, Biomarkers, and Antiplatelet Therapy

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Abstract

The *Journal* provides the clinician and scientist with the latest advances in discovery research, emerging technologies, pre-clinical research design and testing, and clinical trials. We highlight advances in areas of induced pluripotent stem cells, genomics, biomarkers, multi-modality

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imaging and antiplatelet biology and therapy. The top publications are critically discussed and presented along with anatomical reviews and FDA insight to provide context.

Keywords

Anatomy; Anti-platelet; Aortic Valve; Atrial Fibrillation; Biomarker; Genetic; Genomic; Heart Failure; Imaging; Induced pluripotent stem cell; Mitral Valve; Platelet; TAVR

As editors, we aim to provide today's clinician and scientist with novel, accurate, and clinically relevant information with the molecular, cellular and/or genomic mechanism. In this review, the top advances from the year are critically discussed along with anatomical reviews, trial design input from the FDA, and landmark discoveries in the field as reference points. Topic areas include induced pluripotent stem cells (iPSCs), genomics, biomarkers, imaging, and antiplatelet-therapy.

iPSCs

The generation of mouse and human iPSCs from fibroblasts through the transduction of four factors (octamer binding transcription factor 4 (Oct4), sex determining region Y-box 2 (Sox2), Kruppel-like factor 4 (Klf4) and the cellular homolog of the retroviral v-myroncogene (c-Myc)) (1,2), opened a growing area of translational research that includes discovery, drug screening, and therapy. A therapy generating considerable interest is the reprogramming of human fibroblasts for the repair of the failing heart. The *Journal* published an insightful review detailing the methods and limitations for direct reprogramming of human and mouse fibroblasts to cardiomyocytes (3). Published factors necessary for reprogramming of human fibroblasts include Gata-binding protein 4 (GATA4), Mads box transcription enhancer factor 2 polypeptide C (MEF2C), T-box 5 (TBX5), Heart- and neural crest derivatives-expressed 2 (HAND2), myocardin, (MYOCD), and various miRs (3). A major limitation continues to be the low percentage of fibroblasts that actually are reprogrammed to cardiomyocytes (3). The *Journal* also published a study highlighting that reprogramming strategies influence the differentiation capacity of derived iPSCs, supporting the notion of c-Myc-free reprogramming as a strategy to facilitate oxidative metabolism-dependent lineage engagement (4). The *Journal* will continue to publish additional studies in this growing area in the coming year.

A recognized strength of iPSCs is the new approach to modelling disease. Long QT syndrome provides an example of iPSCs being utilized as a model to understand this life threatening disorder (5,6). The rationale for establishing a model *in vitro* for long QT syndrome is based on the clinical significance that patients with a prolonged QT interval are susceptible to a specific ventricular tachycardia that may result in sudden cardiac death (7). The review in the *Journal*, outlines all Long QT syndromes (LQT1-13) along with the affected gene, gene product, and known function of the gene product (7). By reprogramming dermal fibroblasts from patients with these Long QT syndromes, researchers have shown detailed phenotyping of action potentials and calcium trafficking with whole-cell patch clamp, calcium imaging, and extracellular multielectrode recordings (7). Limitations and challenges with these model systems are included (7).

Genomics

Chronic hypertension increases the contractile demands of the heart. These are met by inducing a growth response in cardiomyocytes that results in hypertrophy of the left ventricle. Although initially an adaptive response, left ventricular hypertrophy (LVH) progressively leads to cardiac remodelling and eventually to heart failure. LVH is a complex process that goes beyond mere cardiomyocyte hypertrophy and is characterised by perivascular and interstitial fibrosis, remodelling of the coronary microcirculation and various metabolic changes (8,9). The molecular mechanisms regulating LVH and the different pathological processes associated with heart remodelling are poorly understood. For an overview of the ventricular architecture and the two prevailing models in the literature that describe the movement of the ventricular wall and response to changes in contractile demand the reader is referred to an anatomical review (10).

To define the different components that contribute to LVH, the Petretto group used a rat model of chronic hypertension in which Spontaneously Hypertensive Rats were crossed with Brown Norway to generate a variety of rat strains with different genetic backgrounds and varying degrees of LVH, blood pressure and other pathological parameters (11). The authors found that both LVH and vessel remodelling are independent of blood pressure variation, as they had also previously shown for left ventricular mass (12). LVH, vessel remodelling and also myocardial fibrosis seemed to co-vary together, rather than with blood pressure, suggesting that the changes in these parameters are not secondary to changes in blood pressure and that they are genetically regulated in these animals (11).

Rather than looking for a single gene that would explain the phenotypic changes between the rat strains, the authors looked for networks of co-expressed genes in the heart of these animals. They found a number of networks associated with different pathological features. Interestingly, 61% of these networks were also found in human heart failure, indicating that the co-regulation of genes in many of the networks is evolutionarily conserved (11). Gene networks associated with LVH were different from those associated with blood pressure, suggesting that both pathological processes may be controlled by distinct transcriptional programs (11).

The authors also found one network that was associated with both blood pressure and cardiomyocyte area (11). This network seemed to be linked to the transforming growth factor beta (TGF- β) pathway suggesting that this pleiotropic growth factor plays a role in both the development of cardiac hypertrophy and blood pressure (11). Another network of co-expressed genes showed association with both microvessel remodelling and LVH, indicating that these two processes may be regulated by common mechanisms (11). Interestingly these mechanisms may include a role for the immune response, since many of the genes in the network were associated with inflammation (11). Connective tissue growth factor (CTGF) was the centre of another network linked to cardiomyocyte area (11). The role of CTGF in cardiac hypertrophy and fibrosis has been largely debated (13). Whereas it is considered to be a good marker for fibrosis, it seems to be rather involved in cardiac hypertrophy (14-16). Genes co-expressed with CTGF in this network were enriched for binding sites for the transcription factor myeloid zinc finger 1, (MZF1), suggesting a

possible master regulatory role for this protein in the regulation of cardiac hypertrophy and remodelling.

The associations uncovered by this work point towards conserved mechanisms for the regulation of LVH, microvessel remodelling, cardiac fibrosis and blood pressure. Future studies will integrate these data with additional regulatory layers, such as those provided by microRNAs, long non-coding RNAs and alternative splicing, to provide a full picture of gene expression regulation in the remodelling heart (17-20).

Unlike lower vertebrates, which have a remarkable ability to regenerate several parts of their bodies including the heart, mammals have a more limited regenerative capacity restricted to some of their organs. The inability of the adult mammalian heart to regenerate is likely a main reason underlying the high prevalence of ischemic heart failure. Interestingly, a recent paper showed that neonatal mice are able to regenerate their hearts following an injury (21). This work demonstrated that mouse hearts have a narrow window after birth during which cardiomyocytes can still proliferate and regenerate the organ (21). The molecular mechanisms underlying this process are starting to be unveiled and some strategies are emerging that may help us exploit the potential of this natural ability.

Different cells can contribute to myocardial regeneration. First, new cardiomyocytes can be generated by existing cardiomyocytes as it happens in zebrafish (22). Resident stem/progenitor cells can also contribute to the generation of new cardiomyocytes (23-25). Although the existence of these cells is now generally accepted, there is still debate on how much they contribute to cardiac homeostasis and regeneration. Alternatives to endogenous regeneration include the use of cardiomyocytes derived *in vitro* from embryonic stem cells or induced pluripotent stem cells. Furthermore, different groups have shown the ability to reprogram fibroblasts into cardiomyocytes, as discussed in the section on iPSCs. Different non-coding RNAs regulate these processes (26).

RNAs have emerged as multifunctional molecules whose roles go way beyond carrying instructions to make proteins and ribosomes. We now know that most of our genome is transcribed into different sorts of RNAs with a variety of regulatory functions that determine not only if a protein is made, but also when it is made, which isoform is made and in what amount (17,20). Many of these functions are regulated by RNAs generally classified as non-coding, given they do not carry information to make proteins. Beyond their biological interest, non-coding RNAs are very interesting from a medical point of view since they represent both excellent therapeutic targets, biomarkers and potential tools (18,19,26-30). In a very interesting review in the *Journal*, Hudson and Porrello discuss the role and therapeutic potential that non-coding RNAs have in cardiac regeneration (31).

Different microRNAs (miRs) regulate cardiomyocyte proliferation. Postnatal inhibition of miR-15 delays cardiomyocyte cell cycle arrest and facilitates myocardial regeneration. Similarly, overexpression of miR-590 and miR-199a using adenoviral-associated vectors promoted cardiomyocyte proliferation, regeneration and improved function (32). Similar results were obtained when the miR-17-92 cluster was overexpressed (33). The success of these approaches is very much dependent on new delivery strategies that must target the

right cell type. This is particularly relevant when trying to activate endogenous cardiac progenitor cells. The miR-17-92 cluster increases proliferation of these cells (33). miR-21, miR-24 and miR-221 increase their survival after engraftment in an infarcted heart (34). miR-1 and miR-499, on the other hand, promote their differentiation into cardiomyocytes, at least *in vitro* (35). These miRs, together with miR-133 are also involved in cardiomyocyte differentiation from induced pluripotent stem cells (iPSCs) (36,37) although their use in regeneration therapies has yet to be proven. Enhancement of angiogenesis through manipulation of miR-24, miR-92a, miR-23 or miR-27 is a complementary approach to promote regeneration (38). Finally, fibroblast reprogramming into cardiomyocytes has also been achieved through a microRNA cocktail including miR-1, miR-133, miR-208 and miR-499 (39).

These and other miRs, together with some long non-coding RNAs, will provide potential targets and therapeutic tools that will be helpful for the regeneration of the damaged myocardium in the near future.

Biomarkers

In the last few years, high-throughput screening approaches including sequencing have resulted in a rapid expansion of candidate biomarkers. Two reviews in the *Journal* have championed the vision that panels of miR representing distinct aspects of the diseases will provide a more complete picture of patient health and better enable better-tailored treatments (28,30). A thorough discussion is also provided of the routes by which miRs may be protected from degradation and transported in extracellular fluids via protein miR complexes, HDL miR complexes, exosomes, microvesicles or apoptotic bodies (28). This stability of miRs, together with evidence that regulation of mRNA translation by miRs is critical to cardiac development, homeostasis, and disease, has made them attractive biomarker candidates. The reviews however also highlighted a number of specific hurdles for miR biomarkers to overcome during their translation to clinical practice; these include challenging isolation and detection methods, variation, lack of standardized reporting, and the need for large prospective multicentre validation studies (28,30).

Traditional protein biomarker candidates are also increasing and this will likely continue with the on-going application of high throughput proteomics. Protein markers reflect and discriminate between the multiple molecular pathways that work across the spectrum of heart failure syndromes. A review by Hartupee and Mann suggested that markers of the inflammatory pathways are of particular interest in predicting disease severity, the transition to symptomatic heart failure, and prognosis across heart failure with preserved or reduced ejection fraction and acute decompensated heart failure (40). Conclusions suggest that inflammatory mediators provide surrogates for on-going tissue injury and suggest particular utility with serial sampling and in multi-marker panels that reflect the progressive and multifactorial nature of heart failure (40).

The discovery of new biomarkers and investigation of their cellular origins, functions, and response to (patho) physiological provocation is only the first step in the translation to clinical utility. The FDA provided an important review outlining the criteria that should be

considered when translating biomarkers (41). Clear advice for the design, execution, and interpretation of studies intended for analytical validation, clinical performance evaluation, and clinical outcome assessment is outlined (41). Consideration of their recommendations should strengthen the credibility of translational studies and enhance the ability to extrapolate results.

Imaging

The last year has seen numerous advances in non-invasive cardiac imaging. Multimodality imaging has been used to guide transcatheter valve repair and replacement. Non-invasive imaging is also nearing the ability of invasive mechanisms in defining both cardiac anatomy and physiology. And, finally, non-invasive imaging is in the forefront of biologic imaging and therapeutics for atherosclerosis.

Transcatheter Valves

Aortic stenosis is more commonly being treated by percutaneous approaches in patients that are inoperable or at high surgical risk (42). The Journal published a series in 2013 outlining the anatomy of the arterial valves with color images and a current list of approved valves naming both the Edwards SAPIEN and Medtronic CoreValve as approved for use (42). Ragosta describes the role of multimodality imaging during each phase of the transcatheter aortic valve replacement process (43). Transesophageal echocardiography, multi-detector computed tomography (CT), fluoroscopy, and cardiac magnetic resonance (CMR) have integral roles in the pre-procedural evaluation, procedure performance, and post-procedural assessment. Understanding the advantages and limitations of each imaging modality is critical to proper patient selection, proper valve selection, and valve deployment (43).

Similarly, patients with mitral regurgitation at high operative risk are being treated with percutaneous transcatheter approaches (44). The MitraClip system delivers a clip via a transeptal approach that provides apposition between the anterior and posterior mitral leaflets similar to a surgical Alfieri approach, thus creating a double orifice valve that mitigates regurgitation (44). Careful patient selection in terms of valve pathology is critical to the success of the procedure. Lim describes the use of multimodality imaging not only for patient selection but also for clip delivery (45). Three-dimensional transesophageal echocardiography is particularly useful for appropriate clip delivery and assessment of procedural success (45).

Cardiac Anatomy and Physiology

Invasive coronary angiography has long served as the gold standard for assessing coronary artery lesion severity. A comprehensive review of the coronary anatomy was published by Loukas and colleagues in the *Journal* (46). Fractional flow reserve (FFR) and coronary flow reserve (CFR) measured at the time of cardiac catheterization can provide required quantitative measurements of the lesion severity and effect on downstream flow. Advances in image-based modelling and computational fluid dynamics have allowed for computation of both coronary artery pressure and flow using coronary CT. Zarins et al review the use of CT to calculate FFR_{CT} , a ratio of mean coronary artery pressure to mean aortic pressure, at

every point in the coronary artery tree (47). The clinical applicability of this approach is now being validated.

CT has also emerged as a non-invasive method capable of determining coronary artery anatomy and stenosis severity. The ability of CT to determine lesion severity is compromised in heavily calcified areas, in stented segments and by motion artifacts. Patel et al describes the addition of cardiac CT myocardial perfusion imaging to the anatomical determination of lesion severity (48). This method uses a combination of vasodilator stress with imaging allowing for assessment of not only the anatomical significance of a lesion but also the functional significance of the lesion and has been shown to correlate well with invasive measurements of FFR. Challenges remain in approaches to image acquisition and image interpretation. Once standardized, this technology has the potential to be a cost-effective method of non-invasively determining both coronary anatomy and lesion significance (48).

Abnormal microvascular function in the setting of normal coronary arteries is emerging as an important pathophysiology that is associated with ischemia and increased cardiovascular events. The hallmark of this disease process is abnormal CFR. Ng reviews this pathophysiology in women and describes non-invasive modalities being developed to measure CFR (49). Gan et al describes the use of transthoracic color Doppler echocardiography to measure CFR. This is most easily performed in the left anterior descending artery (50). This may prove to be an important translational method as it can be performed serially in animal models as well as in humans without the use of radiation.

Biologic imaging and Therapeutics

Atherosclerosis is a complex immune-inflammatory disease involving many processes and numerous cell lines. The majority of our knowledge of its pathophysiology comes from autopsy studies and animal models (51-54). Acute coronary syndromes most often originate from thin-capped fibroatheromas, which are composed of large immunologically active necrotic cores with macrophages being the predominant cell type. Vazquez-Figueroa et al demonstrated using intravascular ultrasound with virtual histology IVUS-VH (55), that these lesion types are associated with increased major adverse cardiac event at 12 months in the ATLANTA-1 study (56). Verjans et al reviews the use of magnetic resonance imaging (MRI) and positron emission tomography (PET) to noninvasively assess for plaque activity and inflammation associated with vulnerable lesions (57). MRI assesses plaque inflammation using an iron-based blood pool agent that is phagocytized primarily by macrophages. PET scanning uses primarily glucose-based tracers that can assess for macrophage activity. Other novel PET tracers are being developed that take advantage of markers that are highly expressed in activated mononuclear lines (57).

Ultrasound microbubble contrast agents are gas filled bubbles with a polymer shell that can transit through the circulation with properties very similar to red blood cells and can be imaged by transthoracic ultrasound (58). These agents have previously been used for cardiac cavity visualization and measurement of myocardial blood flow. The shells of these microbubbles can be “decorated” with ligands that target to specific disease states for imaging purposes or delivery of therapeutics. Klivanov describes technical considerations in

the development of specific agents for targeting vascular inflammatory markers such as vascular cell adhesion molecule 1, (VCAM-1), microthrombi on the surfaces of plaque, and bacterial biofilms present in infective endocarditis (59).

Antiplatelet Therapy

ALX-0081 (caplacizumab), is a novel antiplatelet agent represented by a nanobody targeting von Willebrand factor (60). ALX-0081 specifically inhibits platelet adhesion to the vessel wall modulating platelet aggregation and subsequent clot formation without significantly increasing bleeding risk (60). Phase I studies in healthy subjects and stable angina patients undergoing percutaneous coronary intervention (PCI) showed that ALX-0081 was well tolerated, and effectively inhibited pharmaco-dynamic markers. In addition, subgroup analysis showed a significant improvement in peripheral endothelial function in patients receiving ALX-0081 as compared with placebo patients (61). Following these results, a Phase II study was initiated in high-risk acute coronary syndrome patients undergoing percutaneous coronary interventions. Based on its mechanism of action, ALX-0081 is also being developed for the orphan disease indication acquired thrombotic thrombocytopenic purpura.

Thrombin is a potent platelet agonist, and an increased thrombin activity has been reported in patients with acute coronary syndromes (62). Platelet effects of thrombin are mediated by protease-activated receptors (PAR), and PAR-1 is the most important receptor in human platelets (62). Voropaxar is an extensively studied PAR-1 antagonist. Two recent large phase III trials (the TRA 2P TIMI 50 and the TRACER trial) with vorapaxar have been critically discussed by de Souza Brito et al. (63). These studies showed that the antagonism of thrombin mediated platelet activation, on top of current standard antiplatelet therapy, results into a consistent reduction of ischemic events, particularly myocardial infarction. At the same time both trials showed a significant increase in the risk of bleeding. These trials highlight that in order to provide a favorable net clinical outcome, voropaxar should be administrated in appropriately selected patients, based on the clinical setting, the risk of bleeding and of recurrent ischemic events, and also the context of concomitant antiplatelet and antithrombotic treatments.

The appropriate antiplatelet therapy in patients with atrial fibrillation (AF) and heart failure (HF) is not clearly defined and is often difficult to manage. These patients are taking multiple drugs, and are thus likely to be at higher risk of bleeding and/or ischemic events (i.e. stroke). Guo et al. critically appraised the literature focusing on the role of antiplatelet therapy specifically in patients with atrial fibrillation and/or heart failure (64). Aspirin has a limited role in stroke prevention among most patients with AF, being of limited efficacy and not any safer than warfarin, especially in the elderly. New oral anticoagulants provide more efficient and safe thrombo-prophylaxis in AF patients without anticoagulation monitoring, which will weaken the role of aspirin in stroke prevention in AF. For HF patients with AF, cardioembolism is a major cause of stroke, and antiplatelet alone would be insufficient for thrombo-prophylaxis. These authors recommended use of dual antiplatelet therapy for those with AF and HF who refused other forms of anticoagulation.

Conclusions

In summary, the field of iPSCs made significant progress in defining multiple cocktails capable of reprogramming both murine and human fibroblasts into cardiomyocytes (3). Genetic mouse models and further understanding of temporal and spatial cues provided by the matrix will be important as we move forward. How this science translates to therapy for the failing heart is yet to fully unfold. The *Journal* placed a focus on RNA in the special issue on Genomics in 2013. This led to an especially high impact paper on non-coding RNA and its role in heart failure and development (19). RNA also is moving rapidly into the field of biomarkers (28). A publication from the FDA provided a clear roadmap of criteria to be considered when translating biomarkers (41). Aortic valves were one of the most significant areas in imaging in the past year. The *Journal* critically discusses these advances from both an anatomical as well as an interventional point of view (42,43). Finally, top publications in antiplatelet therapy included a thorough and insightful look at the new challenges in patient management (64), novel clinical trials (60) and a comprehensive state-of-the-art review on platelet biology (62).

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Abbreviations

AF	atrial fibrillation
CMR	cardiac magnetic resonance
CFR	coronary flow reserve
c-myc	cellular homolog of the retroviral v-myconcogene
CT	multi-detector computed tomography
CTGF	connective tissue growth factor
FFR	fractional flow reserve

GATA4	Gata-binding protein 4
HAND2	Heart- and neural crest derivatives-expressed 2
HF	heart failure
iPSCs	Induced pluripotent stem cells
KLF4	Kruppel-like factor 4
LVH	Left ventricular hypertrophy
MEF2C	Mads box transcription enhancer factor 2 polypeptide C
miR	microRNA
MYOCD	myocardin
MZF1	myeloid zinc finger 1
OCT4	octamer binding transcription factor 4
SOX2	Sex determining region Y-box 2
TBX5	T-box 5
TGF-β	transforming growth factor beta