Developing insights into cardiac regeneration

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Summary

Owing to its intrinsic beauty and biomedical importance, the heart has been the focus of intensive research. The recent EMBO/EMBL-sponsored symposium 'Cardiac Biology: From Development to Regeneration' gathered cardiovascular scientists from across the globe to discuss the latest advances in our understanding of the development and growth of the heart, and application of these advances to improving the limited innate regenerative capacity of the mammalian heart. Here, we summarize some of the exciting results and themes that emerged from the meeting.

Key words: Heart, Development, Regeneration, Cardiac progenitor cells, Transcriptional regulation

Introduction

The heart is one of the first organs to function, and continuous pump function is essential for fetal and postnatal life. The vertebrate heart forms as a single tube, which then loops, expands chambers, and septates to acquire its mature organization. The heart grows rapidly through fetal and early postnatal life. Fetal heart growth is achieved by cardiomyocyte (CM) proliferation, but postnatal mammalian CMs lose their capacity to proliferate, and postnatal growth occurs primarily by increasing CM size rather than number. Diseases such as myocardial infarction (MI) result in the loss of billions of CMs leading to heart failure, for which we lack specific therapies. The profound medical and economic impact of heart disease has led cardiovascular scientists to focus on the mechanisms that regulate heart growth and development and to use this knowledge to design approaches for cardiac regeneration.

In June 2013, the EMBO/EMBL symposium 'Cardiac Biology: From Development to Regeneration' brought together a diverse group of over 200 cardiovascular scientists from six continents, among them many of the current and future investigators at the forefront of this rapidly developing field. The meeting was organized by Nadia Rosenthal (EMBL Australia, Australia), Robert Graham (Victor Chang Cardiac Research Institute, Australia), Stephanie Dimmeler (Goethe-University Frankfurt am Main, Germany) and Didier Stainier (Max Planck Institute for Heart and Lung Research, Germany) and was held at the magnificent EMBL Advanced Training Centre in Heidelberg, Germany. The meeting featured wide-ranging topics that highlighted many of the most recent developments in the field, with time for thought-provoking questions and many opportunities for discussions among conference participants. An overarching theme of the meeting was how understanding heart development is the foundation for advancing towards the goal of cardiac regeneration (Fig. 1). Here,

we highlight some of the outstanding talks, advances and interesting questions from the meeting.

Heart development

An important question in cardiac biology is whether cardiac lineages are plastic, i.e. whether differentiated cells of a particular lineage can transdifferentiate to another lineage. Didier Stainier showed that ablation of ventricular cells in 3-4 days post-fertilization (dpf) larvae leads to subsequent regeneration. Strikingly, lineage tracing of atrial cells labeled prior to ventricular ablation showed that regenerated ventricular cells appeared to derive from atrial cells that were reprogrammed (Zhang et al., 2013). Inhibiting Notch signaling blocked this transdifferentiation. These observations indicate plasticity of cardiac lineage-specific differentiated cells in the embryo in the context of myocardial injury.
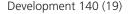
Stainier also described the process of cardiac trabeculation *in vivo* at the cellular level using high-resolution 3D time-lapse imaging (4D imaging) of beating zebrafish hearts. The combination of 4D imaging and analysis of *erbb2* null genetic mosaics (Liu et al., 2010) illustrated the dynamic movement of CMs whereby the cells extend processes that invade at the luminal side into the forming trabecular layer and subsequently reposition to contribute to the trabecular layer. His data also showed that cardiac contraction might also promote cardiac trabeculation, because genetic or pharmacological paralysis of the heartbeat blocked trabeculation.

That fluid forces play important roles in organ morphogenesis was further illustrated by Nadia Mercader (National Center for Cardiovascular Research, Spain). Using high-speed imaging and optical tweezing in zebrafish embryos, Mercader showed that heartbeat-driven pericardial fluid forces trigger proepicardium formation at several sites within the pericardial wall, modulate epicardial progenitor cell release and motion, and determine the location of their adhesion to the myocardial wall. These studies, which were accomplished as part of a collaboration with the group of Julien Vermot (Institute of Genetics and Molecular and Cellular Biology, France), represent the first example of extra-cardiac flow forces controlling cardiogenesis.

The heart is the first organ to develop left-right asymmetry. Cecilia Lo (University of Pittsburgh, USA) provided an update on a mouse N-ethyl-N-nitrosourea (ENU) mutagenesis screen in which 150 mutant lines with a wide spectrum of structural congenital heart defects (CHDs) were recovered, including the first mouse models of hypoplastic left heart syndrome. Strikingly, almost half of the lines exhibited complex heart defects in association with laterality defects. Exome sequencing revealed mutations in cilia genes not only in many lines with laterality defects, but also in a significant fraction of lines with CHDs but no laterality defects. These findings suggest a role for cilia defects in CHDs independent of the important role of cilia in establishing left-right asymmetry.

The theme of left-right asymmetry continued with Salim Seyfried (Max Delbrück Center for Molecular Medicine, Germany)

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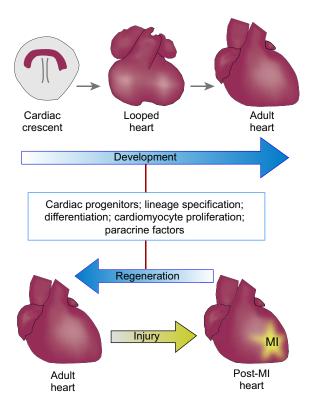*Authors for correspondence (v.m.christoffels@amc.uva.nl; wpu@pulab.org)

Fig. 1. Lessons from heart development inform efforts to promote cardiac regeneration. Studies of heart development have yielded biological insights that may be effectively deployed to regenerate the heart after myocardial injury.

who discussed the role of bone morphogenetic protein (BMP) signaling in modulating cell motility and thereby directing cardiac left-right asymmetry (Veerkamp et al., 2013). Left-sided Nodal reduces BMP signaling specifically at the left side of the cardiac field of zebrafish embryos. Reduced BMP signaling resulted in lower expression of non-muscle myosin II and higher cell motility on the left. Computational modeling indicated that this difference in cell motility is sufficient to induce directional migration of cardiac tissue to the left, which is the first step in establishing cardiac left-right asymmetry. These studies provide a mechanistic link between anti-motogenic BMP activity and cardiac left-right asymmetry.

Epigenetic and transcriptional regulation

Heart development is regulated by gene regulatory networks consisting of a set of highly conserved tissue-specific transcription factors, signaling molecules and non-coding RNAs. Central to this network are the transcription factors NKX2-5, GATA4 and SRF, which, together with their target DNA elements, form an evolutionarily conserved subcircuit essential for development, dubbed a 'kernel' (Davidson and Erwin, 2006). Richard Harvey and Mirana Ramialison (Victor Chang Cardiac Research Institute, Australia) studied this kernel by comparing the target sites of wild-type NKX2-5 with those of a mutated form of NKX2-5 in which the homeodomain is missing. Off-target sites for mutant NKX2-5 were identified that were enriched in motifs for ubiquitous transcription factors, including members of the ETS family, which was attributed to interaction between mutant NKX2-5 and ETS domain-containing proteins. This suggested that ubiquitously

expressed factors might be embedded in the heart kernel. Further light was shed on gene regulation by Silke Sperling (Charité Universitätsmedizin, Berlin; and Max Delbrück Center for Molecular Medicine, Germany) who discussed the crosstalk between cardiac transcription factors, chromatin remodeling enzymes and histone modifications. Some redundancies in the network became apparent and particular transcription factors, such as GATA4 and NKX2-5, co-occupied target sites, implicating the preferential interactions of these factors with chromatin remodeling enzymes, such as BAF60 and its associated SWI/SNF complex.

The architecture of the chromatin is an important aspect of gene regulation and thus of the gene regulatory networks. Vincent Christoffels (Academic Medical Center, Amsterdam) used 4C-seq, a technique that probes three-dimensional chromatin architecture to enhance dissection of the regulatory circuitry of *Tbx3*, a crucial component of the gene regulatory network for the cardiac conduction system (CCS) (van den Boogaard et al., 2012). Sites of interaction between putative regulatory elements and the *Tbx3* promoter appeared to be limited to the region between the flanks of the *Tbx3* locus. *In vivo* enhancer analysis showed that these sites harbor evolutionarily conserved *Tbx3* enhancers that are necessary and sufficient to drive expression in the CCS, recapitulating the *Tbx3* expression pattern. These data provide an example of the involvement of chromatin 3D architecture in the regulation of developmental gene expression.

Margaret Buckingham (Institut Pasteur, France) discussed the transcriptional pathways that control activation of fibroblast growth factor 10 (*Fgf10*), a key gene regulating the behavior of secondary heart field progenitors, which contribute CMs to the ends of the growing heart tube. Buckingham's work investigated transcriptional pathways that regulate *Fgf10*, a gene known to regulate the anterior second heart field (SHF) progenitors. She showed that *Fgf10* expression depends on an enhancer regulated by TBX1, NKX2-5 and ISL1 (Watanabe et al., 2012). The enhancer acts as a switch that is activated in the SHF by high levels of ISL1 and TBX1 and is repressed in myocardium by high levels of NKX2-5, which acts both directly and indirectly through *Isl1* suppression. This example provides a paradigm for changes in regulatory networks during the transition from the progenitor state to that of differentiated myocardium.

Non-coding RNAs have been implicated in various gene regulatory networks across a vast number of developing and mature organ systems, and the heart is no exception. Bernhard Herrmann (Max Planck Institute for Molecular Genetics, Germany) and Stefanie Dimmeler both presented evidence for the role of non-coding RNAs in the developing and aged heart, respectively. Herrmann presented work on *Fendrr*, a tissue-specific long non-coding RNA (lncRNA) that is transiently expressed in the lateral mesoderm that gives rise to the heart and body wall. Inactivation of *Fendrr* in mouse revealed its requirement for heart and body wall development (Grote et al., 2013). *Fendrr* interacts with both polycomb and trithorax epigenetic regulatory complexes and the double-stranded DNA (dsDNA) of target promoters, suggesting that it recruits these antagonistic complexes to different promoters in order to modulate their epigenetic landscape and to regulate target gene expression in the developing heart. Dimmeler discussed the function in the adult of microRNA-34a, which is induced in the aging heart (Boon et al., 2013). Deletion or reduction of miR-34a reduced the CM cell death and fibrosis caused by myocardial ischemia or aging. PNUTS (also known as PPP1R10), a protein that reduces both telomere shortening and the DNA damage response, was identified as a key target of miR-34a.

Heart regeneration from endogenous cell sources

A fundamental question facing cardiac biologists is the extent to which CMs proliferate in the normal heart. Ahsan Husain (Emory University, USA) described a meticulous study of juvenile murine heart growth, which identified an unexpected spike in proliferative activity of binucleated CMs on post-natal day (P)15, suggesting that these cells might not be terminally differentiated. Moving on to humans, Olaf Bergmann (Karolinska Institute, Sweden) presented a study on CM proliferation in pediatric patients and adult humans based on analysis of human hearts using unbiased stereology. Measurement of both CM volume and CM nuclear density indicated that CM number did not change substantially in young humans. However, Bernhard Kuhn (Boston Children's Hospital, USA) investigated the same question and reached a different conclusion (Mollova et al., 2013). Measurement of CM volume and numbers, karyokinesis, cytokinesis and ploidy showed that the CM number increases by 3.4-fold over the first 20 years of life, indicating that heart growth in young humans occurs through a combination of an increase in CM number and size. Given these apparently contradictory results, further work is clearly required to determine conclusively the degree of proliferation and timing of terminal CM differentiation in the postnatal heart.

The capacity of the postnatal heart to regenerate in normal and injury conditions has also been investigated in mouse models. Eric Olson (University of Texas Southwestern Medical Center, USA) previously reported that the mouse is able to regenerate resected myocardium up to P7 (Porrello et al., 2011). The type of injury probably influences the regenerative response, as Olson showed that regeneration occurs after neonatal myocardial infarction, whereas Bernhard Kuhn indicated that the neonatal mouse heart fails to regenerate effectively after cryoinjury. Interestingly, neonatal myocardial regeneration appears to require an initial inflammatory response involving macrophages, because depletion of macrophages using clodronate liposomes nearly completely blocks the neonatal heart regeneration after MI.

The involvement of macrophages in heart and skeletal muscle regeneration was further expanded upon in presentations by Nadia Rosenthal and Thomas Braun (Max Planck Institute for Heart and Lung Research, Germany). Braun reported on newt heart regeneration; explant studies revealed that mechanically injured newt hearts must remain *in situ* for at least a few days in order to regenerate. If the hearts are explanted immediately following injury, they fail to regenerate effectively. At least one reason for this is the need for macrophage infiltration, as macrophage depletion by clodronate liposomes also prevented newt heart regeneration. Braun described an additional positive-feedback loop involving macrophages that functions to minimize injury in the adult mouse heart after MI. Oncostatin M, secreted by CMs from injured myocardium, recruits macrophages. These macrophages then secrete Reg3b, a cytokine that stimulates further macrophage recruitment. Forced inactivation of Reg3b compromised heart function after MI and at the same time impaired scar formation, resulting in higher risk of cardiac rupture. Rosenthal also reported a role for macrophages in axolotl limb regeneration (Godwin et al., 2013), in which macrophage infiltration occurs early after limb amputation and is necessary for regeneration. Rosenthal then went on to identify a putative role for Treg anti-inflammatory cells in insulin-like growth factor (IGF)-mediated scar-free healing in skeletal muscle.

Strategies for stimulating endogenous heart regeneration or minimizing heart injury were presented. One approach is to enhance CM proliferation. William Pu (Boston Children's Hospital, USA) and Eric Olson reported independent studies showing that YAP1 is sufficient to drive adult CM proliferation and to improve survival and myocardial outcome after MI. Felix Engel (Universitätsklinikum Erlangen, Germany) described a screen to identify small molecules that enhance neonatal CM proliferation. One compound identified was carbacyclin, which stimulates PPARδ to activate β-catenin signaling. Despite the identification of signaling and transcriptional pathways that stimulate CM proliferation in neonatal CMs, adult CMs are strikingly resistant to proliferation. David Zebrowski, a member of the Engel lab, showed that at least one fundamental block results from loss of centriole cohesion in mature binucleated cardiomyoytes and consequent activation of a centriole-mediated mitotic checkpoint.

Kenneth Chien (Karolinska Institute, Sweden) described a novel approach to minimizing heart injury, based on a therapeutic paradigm of delivering a paracrine signal at the right time and place to modulate cardiac progenitor cell fate. He reported that modified RNA encoding VEGFA given at the time of MI delivers a strong VEGFA pulse that lasts ~48 hours. This pulse augments epicardial progenitor function, mobilizes them from the heart surface, and directs their differentiation towards the endothelial lineage, thereby improving myocardial perfusion, function and long-term survival.

Eric Olson summarized his group's progress on *in vivo* reprogramming of fibroblasts to CMs (Ieda et al., 2010; Song et al., 2012). *In vitro*, his lab found that forced expression of the major cardiac transcription factors GATA4, HAND2, MEF2c and TBX5 (GHMT) reprogram tail tip mouse fibroblasts into CMs. *In vivo*, this process is considerably more efficient and leads to long-term enhancement of heart function after MI. His lab has made progress in identifying small molecules that significantly enhance the efficiency of the reprogramming process, and by finding that human fibroblasts can be reprogrammed using GHMT plus myocardin.

Building a heart: stem and progenitor cells and their application to heart regeneration

Other potential approaches to cardiac regeneration involve augmenting the responses of endogenous cardiac progenitors, or delivering progenitor cell populations or their derivatives to the infarcted heart after amplification *in vitro*. To modulate endogenous cardiac progenitor populations, it is first necessary to define these populations. This goal has been both elusive and contentious. Michael Schneider (Imperial College London, UK) described his efforts to characterize the murine Sca1 (Ly6a – Mouse Genome Informatics)-positive heart progenitor population (Oh et al., 2003) and showed that the progenitor subset can be substantially enriched by sorting for either high efflux of Hoescht dye ('side population') or by high expression of PDGFRa. The enriched population expresses various combinations of the cardiac transcription factors GHMT and has multi-lineage potential in cardiac grafts. Bernardo Nadal-Ginard (King's College London, UK) described an alternative cardiac progenitor cell population in mice characterized by expression of the marker c-Kit (Kit – Mouse Genome Informatics) (Beltrami et al., 2003). He reported a clever and demanding set of experiments that led him to conclude that c-Kit^{+} cells are necessary and sufficient to drive regeneration in an acute adult myocardial injury model based on a cardiotoxic dose of isoproterenol (Ellison et al., 2007).

There has been tremendous interest in delivering progenitor cells or mature *in vitro* differentiated CMs into the heart to drive heart repair. Charles Murry (University of Washington, USA) described a Herculean effort in his lab to provide proof of concept that human

embryonic stem cell (ESC)-derived CMs can be safely transplanted and electrically integrated into the heart of a non-human primate after MI. His group delivered 10^9 human ESC-derived CMs into the two-week-old infarcted heart of immunosuppressed *Macaca nemestrina*. Initially, the treatment induced cardiac arrhythmias, including ventricular tachycardia and accelerated idioventricular rhythm, but this pro-arrhythmic effect resolved after about two weeks, by which time electrical coupling was observed between graft and native myocardium. Grafts appeared to be perfused by a sluggish vascular plexus, and developing a brisk arterial supply is a substantial remaining hurdle. Lior Gepstein (Technion-Israel Institute of Technology, Israel) touched on his lab's efforts to meet this challenge using engineered biomaterials and a mixture of CM, endothelial and mesenchymal cells to generate grafts with improved vascularization.

More than 2600 patients have already received bone marrow-derived mononuclear cell (BMC) transplantation for ischemic heart disease (Jeevanantham et al., 2012). Although injected bone marrow-derived cells do not persist in host tissue, their transient homing to injured myocardium appears to exert paracrine and immunomodulatory effects that may enhance myocardial recovery from infarction. Andreas Zeiher (Goethe University Hospital, Germany) reviewed his group's experience with this form of cell therapy. In patients with only modestly reduced ejection fraction, BMC cells showed little benefit. However, in patients with more severely reduced ejection fraction (<45%), BMC-treated patients had reduced adverse remodeling and better preservation of heart function. Individual patient outcomes were highly heterogeneous, however, and one important factor might be the homing efficiency of the injected BMCs. Zeiher showed data that this might be improved by echocardiography-guided shock wave pretreatment to enhance myocardial release of chemotactic factors.

Paul Simmons (Mesoblast, Australia) discussed Mesoblast's evaluation of bone marrow-derived, immunopurified mesenchymal precursor cells for cardiovascular indications. In an ovine MI model, intracardiac delivery of these cells resulted in better cardiac ejection fraction at 8 weeks, largely due to paracrine and immunomodulatory effects. Among the many factors excreted by these cells are HGF, IGF1, IL6, MCP1, SDF1 and VEGF. Although there may be reason to believe that BMC therapy has a beneficial effect in subsets of patients with MI, the mechanisms underlying this phenomenon require further investigation.

Modeling heart disease with induced pluripotent stem cells (iPSCs)

Our improving understanding of stem cell biology, cardiogenesis and CM function have converged upon developing *in vitro* models for human disease. Of great value in this respect are induced pluripotent stem cells (iPSCs), which can be derived from patients with a variety of diseases and differentiated to CMs to recapitulate disease phenotypes and mechanisms. Joseph Wu (Stanford, USA) summarized his lab's efforts in using iPSCs derived from patients with dilated and hypertrophic cardiomyopathy to model core features of these diseases (Sun et al., 2012; Lan et al., 2013). He also outlined the value of iPSC-derived CMs for screening of new drug candidates for cardiotoxicity, and presented a vision in which this screening is performed against a bank of 1000 iPSC-derived human CMs to capture human genetic variation and perhaps identify subgroups in which a drug might be safe or unsafe. Lior Gepstein discussed his group's experience using patient-derived iPSCs to model long QT syndrome and arrhythmogenic right ventricular cardiomyopathy (ARVC) (Itzhaki et al., 2011). He observed that the CMs

differentiated from ARVC iPSCs accumulated lipid droplets, which were reminiscent of fatty replacement seen in patients, and this was linked to increased activity of PPARγ in the disease cells.

However, *in vitro* models continue to have limitations, most notably the immature phenotype of current iPSC-derived CMs. Malin Jonsson Boezelman (Genome Institute of Singapore, Singapore) pointed out the importance of this problem in the context of using pluripotent stem cell-derived CMs for cardiotoxicity assays. These CMs lack or have low levels of some key ionic currents present in adult CMs, and consequently these screens can yield both false-positive and false-negative results. Developing appropriate conditions to mature properly *in vitro* differentiated CMs is necessary to overcome this limitation.

Conclusion

This meeting summary highlights some of the exciting advances being made in cardiovascular biology. Using development as a roadmap towards regeneration has brought us to a point at which clinical therapy can be contemplated and even implemented in some cases. However, we also learned that the heart does not yield its mysteries easily. We still have a long way to go to realize the vision of cardiac regeneration, and insights from developmental biology will surely light the way.

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