© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 1435-1441 doi:10.1242/dev.133652

MEETING REVIEW

Heartbreak hotel: a convergence in cardiac regeneration

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ABSTRACT

In February 2016, The Company of Biologists hosted an intimate gathering of leading international researchers at the forefront of experimental cardiovascular regeneration, with its emphasis on 'Transdifferentiation and Tissue Plasticity in Cardiovascular Rejuvenation'. As I review here, participants at the workshop revealed how understanding cardiac growth and lineage decisions at their most fundamental level has transformed the strategies in hand that presently energize the prospects for human heart repair.

KEY WORDS: Cardiomyocyte, Heart, Regenerative medicine, Stem cells

Introduction

The first organ to be fashioned during embryogenesis – the heart – is the organ that contributes most, worldwide, to death, disability and healthcare system costs (Roth et al., 2015). This daunting global burden is due most often to coronary artery obstruction, but is also compounded by inbuilt biological limitations that constrain the renewal of human cardiomyocytes, preventing effective self-repair. No approved therapy targets cardiac regrowth, making urgent the case to explore regenerative medicine approaches. The publishers of Development recently hosted a workshop entitled 'Transdifferentiation and Tissue Plasticity in Cardiovascular Rejuvenation', which was organized by Brian Black (University of California, San Francisco, USA) and James Martin (Baylor College of Medicine, Houston, USA), aiming to provide developmental perspectives on cardiac regeneration and repair. Rather than dwell on past controversies, the participants (Fig. 1) shared a prudent perspective that no therapy now in human trials was likely to be optimal, and that a panoply of development-led remedies is yet to be unleashed.

Cardiac progenitors and stem cells

Specification of the cardiac lineage during gastrulation in mouse embryos was addressed by Elizabeth Robertson (University of Oxford, UK). The Nodal, BMP and Wnt3 pathways are essential to induce nascent mesoderm in the primitive streak (Sampath and Robertson, 2016). A key target of Nodal and its receptor-activated effector, phosphorylated Smad2/3, is the T-box gene eomesodermin (*Eomes*), and the recent fate mapping of *Eomes*-derived cells (Costello et al., 2011) suggests that it is crucial for the emergence of both definitive endoderm and cardiovascular progenitors. Robertson described how one transcription factor activated directly by Eomes – *Lhx1* – is required for normal midline and head structure morphogenesis, yet is dispensable for the heart (Nowotschin et al., 2013; Costello et al., 2015). By contrast, *Mesp1* and *Mesp2* are direct Eomes targets that are crucial for cardiovascular development. She further showed that Eomes, along with Tbx6, binds an essential early mesodermal enhancer shared by *Mesp1* and *Mesp2*, and deletion of this element prevents *Mesp1/2* activation, the epithelial-mesenchymal transition, and induction of the cardiovascular progenitors.

Cardiovascular origins were also discussed by Christian Mosimann (University of Zurich, Switzerland). In vertebrates such as zebrafish, lateral plate mesoderm (LPM)-derived cell types include not only the heart, but also blood vessels, blood, kidney and pectoral fins. Mosimann described how a 6.5 kb fragment of the zebrafish *draculin* (*drl*) upstream region initially labels all LPM derivatives but becomes restricted just to cardiovascular and hematopoietic lineages and, later still in the heart, just to first heart field (FHF) descendants (Mosimann et al., 2015). Further dissection of the *drl* locus revealed a pan-LPM enhancer plus anterior and posterior LPM enhancers, while *drl* reporter zebrafish showed that regulators of cardiac septation control the heart field contributions, with loss of *pitx2* diminishing the FHF and loss of *tbx5* expanding it (Mosimann et al., 2015). Using panoramic lightsheet microscopy of *drl* reporters, combined with *sox17*-based endoderm labeling, the stepwise specification of the LPM from mesendoderm could be documented. Intriguingly, the *drl* pan-LPM enhancer drives reporter activity in different model organisms, suggesting that a conserved upstream program defines the initial LPM and sets the stage for subsequent cardiovascular development.

Turning to cardiac progenitors in adult hearts, Michael Schneider (Imperial College London, UK) investigated dormant cardiac progenitor/stem cells identified in adult mice initially on the basis of stem cell antigen 1 (Sca1; Ly6a) expression (Oh et al., 2003) and refined according to their side population (SP) dye-efflux phenotype (Matsuzaki et al., 2004) and PDGFRα expression, features that pinpoint the clonogenic cardiogenic Sca1$^+$ cell (Noseda et al., 2015a,b). The cloning efficiency of fresh PDGFRα$^+$ SP cells in physiological oxygen is a noteworthy 30%. Single-cell progeny show multi-lineage potential after cardiac grafting, with well-organized sarcomeres and incorporation into blood vessels at 12 weeks. However, durable engraftment is rare, implicating early paracrine effects as most credible for the large benefits observed (reduced scar size, improved pump function, reduced dilatation). Schneider further described how a microgroove-patterned substrate, investigated in collaboration with Molly Stevens (Imperial College London, UK), enhances the programming of adult cardiac stem cells toward a cardiomyocyte fate (Morez et al., 2015). These results highlight the impact of purely physical signals on cardiac stem cell function.

Richard Harvey (Victor Chang Cardiac Research Institute, Sydney, Australia) discussed cardiac mesenchymal/stromal cells that resemble the archetypal mesenchymal stem cell in bone marrow, using the colony-forming assay pioneered almost 50 years ago (Friedenstein et al., 1970). The resulting cardiac colony-forming unit-fibroblast (cCFU-F) (Chong et al., 2011) has broad mesodermal plasticity and even tri-germ-layer potential in culture. Harvey showed that the starting Sca1$^+$ PDGFRα$^+$ CD31 (Pecam1)$^-$

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Development (2016) 143, 1435-1441 doi:10.1242/dev.133652

Fig. 1. Group photograph.

cells (S^+P^+) from which the colonies arise are perivascular, but based on their anatomical position are not pericytes. Flow cytometry with pyronin Y, a marker of RNA content, demonstrated that many of these cells are quiescent in healthy hearts but activated following cardiac injury. Parabiosis of old mice with young mice rescued the growth properties of S^+P^+ cells from older hearts. Encouraging evidence that endogenous S^+P^+ cells might be a rational target for therapeutic modulation was their enhancement by PDGFA/B infusion, accompanied by reduced scar size and improved cardiac function.

Together, these studies of cardiac myogenesis in the embryo and later cardiac progenitors reveal the complexities of patterning the *Eomes*-specified mesoderm, but also the importance of secretory signals both to and from the cardiac stem cell.

Lymphangiogenesis in the cardiovascular system and beyond

Paul Riley (University of Oxford, UK) discussed the development of cardiac lymphatic vessels. Historically, two views have contended: that lymphatics arise wholly by budding off from veins, or instead arise from mesenchymal cells (Semo, et al., 2016). Riley described how cardiac lymphatics, marked by *Vegfr3* (*Flt4*) and *Prox1*, develop only in part from the common cardinal vein, a portion being *Tie2* (*Tek*) negative (not venous derived). Further fate mapping excludes the pro-epicardial organ, cardiac mesoderm and cardiac neural crest as the source of these unexplained lymphatic endothelial cells, pointing instead to hemogenic endothelium (Klotz et al., 2015). Cardiac injury induces neo-lymphangiogenesis, inducing the developmental program (*Vegfr3*, *Lyve1*, *Prox1*) and extensive lymphangiogenic sprouting after myocardial infarction. This neo-lymphangiogenesis can be augmented by recombinant VEGFC during cardiac self-repair, reducing infarct size and improving ventricular performance (Klotz et al., 2015).

Karina Yaniv (Weizmann Institute, Rehovot, Israel) likewise studied lymphangiogenesis. Fate mapping with the photoconvertible protein Kaede expressed in zebrafish endothelial cells showed that more than 90% of lymphatic progenitors arise from the ventral posterior cardinal vein (PCV), in close contact with endoderm (Nicenboim et al., 2015). However, ventral PCV cells are heterogeneous, also generating arteries and veins, indicating that they are angioblasts, not venous cells. Imaging the cardiac lymphatics with *fli:egfp* in addition to *lyve1:dsred* and *prox1:RFP* revealed that the vessels sprout from the outflow tract and are indeed heterogeneous in origin. PCV-specialized angioblasts also provide a common origin for all vessels of the subintestinal plexus – the vessels that vascularize the gut, liver and pancreas (Hen et al., 2015).

Driving cardiomyocyte proliferation in the embryo, neonate and adult

In zebrafish (Foglia and Poss, 2016) and newborn mice (Porrello and Olson, 2014), new cardiomyocytes are created after injury and culminate in scarless healing via the proliferation of pre-existing myocytes. Ken Poss (Duke University, Durham, USA) described his group's efforts to find regulatory elements that control such regeneration in zebrafish. A transcriptomic analysis comparing heart and fin regeneration identified hundreds of genes in common, from which some of the most highly induced genes during regeneration were exploited to identify DNA regions with areas of chromatin that open during heart regeneration. Poss described ongoing work to examine whether elements identified from these analyses could drive the expression of reporter genes or mitogens after injury as a potential means to modify regenerative capacity.

Also using zebrafish, Nadia Mercader (University of Bern, Switzerland) examined the interplay between fibrosis and regeneration during heart repair. Fate mapping and genetic ablations were used to study the contribution of different myocytes to repopulating the injured heart, as well as their requirement for extracellular matrix components during myocyte proliferation. By contrast, Juliane Münch (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) followed the

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endocardium during zebrafish heart regeneration, distinguishing a pathway that promotes endocardial organization after injury and helps terminate inflammation.

Interactions between endocardial and myocardial cells were highlighted by Didier Stainier (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany), who presented elegant studies of trabeculation – the inward growth of cardiac muscle from the outer compact layer. Using *LIFEACT:GFP* to label filamentous actin, Stainier demonstrated that trabeculation in zebrafish mostly occurs by delamination and not via oriented cell division (Liu et al., 2010). Trabeculae fail to form in zebrafish lacking *erbb2*, which encodes a Neuregulin receptor, and transplanted *erbb2* mutant cells fail to incorporate into wild-type trabeculae. The trabeculation defect is recapitulated in mutants lacking a specific Neuregulin ligand, which is expressed with striking periodicity near the delaminating cells. Also impacting on cardiomyocyte development are flow-responsive genes described to be expressed in endocardial cells: in zebrafish mutants for some of these genes, cardiomyocytes are aberrantly extruded to the abluminal side, further illustrating the complex signaling between endocardial and myocardial cells.

Eldad Tzahor (Weizmann Institute of Science, Rehovot, Israel) also focused on the Neuregulin/ErbB2 pathway (D'Uva et al., 2015), as well as on features of the cardiomyocyte extracellular microenvironment (Yahalom-Ronen et al., 2015). Levels of ErbB2 fall sharply in mouse myocardium in the first week after birth, suggesting that its loss might mediate the postnatal loss of proliferative capacity. Indeed, Tzahor showed that the cardiomyocyte-specific deletion of *Erbb2* provokes a hypoplastic heart; conversely, even when induced in young adults, 'Tet-Off' transgenic expression of activated *Erbb2* drives myocyte cycling, mitosis and cytokinesis, enhancing regenerative growth and function (D'Uva et al., 2015). Intriguingly, the team identified a component of the P1 (but not P7) matrix that promotes cardiomyocyte proliferation. Using mass spectrometry, they identified the proteoglycan Agrin and showed that it is essential for myocyte proliferation and neonatal heart repair, and sufficient to boost cardiac regeneration in juvenile and adult infarct models.

The role of the Hippo pathway in regulating cardiomyocyte proliferation was discussed by James Martin (Baylor College of Medicine, Houston, USA). The scaffold protein, Salvador, is essential for normal Hippo signaling, and Martin discussed how its deletion in the developing mouse heart unleashes cardiomyocyte proliferation (Heallen et al., 2011). Analogously, its deletion in the adult heart with tamoxifen-dependent Cre enables adult myocytes to proliferate robustly and execute effective heart repair (Heallen et al., 2013). ChIP-Seq and expression profiling of Hippo-deficient hearts showed that the genes targeted by Yap, the Hippo pathway transcriptional co-activator, encode cell cycle components, as expected, but also notably proteins for actin polymerization and its linkage to the extracellular matrix (Morikawa et al., 2015). Strikingly, Hippo-deficient myocytes extend unique sarcomere-filled protrusions and are highly invasive *in vitro*. Hippo-deficient hearts also show a number of fetal characteristics that appear to be important for Yap-mediated regenerative potential. Thus, any future treatment strategy is likely to need to finely calibrate Hippo signaling levels.

Ahmed Mahmoud (Harvard Medical School, Boston, USA) explored the phenomenon that cardiomyocyte proliferation after injury depends on nerve activity. Zebrafish were subjected to apical cardiac resection and treated with pharmacological blockers (Mahmoud et al., 2015). Whereas the beta-adrenergic antagonist propranolol had no effect, cardiomyocyte proliferation was blocked by cholinergic inhibitors or genetic reduction of cardiac innervation (cardiomyocyte-targeted expression of *semaphorin 3Aa*). Recapitulating this phenotype in neonatal mice, cardiomyocyte proliferation was impaired not only by atropine but also by surgical denervation, which caused the dysregulation of diverse genes for immune responses and wound healing, potentially linking this neural pathway to inflammatory mechanisms for neonatal mammalian heart repair (Aurora et al., 2014). Serum from neonatal mice but not adult mice also had a mitotic impact, suggesting systemic contributions from circulating factors. In this context, Andrea Mattiotti (University of Amsterdam, The Netherlands) noted that epicardial patches seeded with recombinant Fstl1 were recently reported to drive cardiomyocyte cycling, suppress fibrosis, and improve cardiac pump function (Wei et al., 2015). Given that the germline deletion of *Fstl1* is lethal at birth (Sylva et al., 2011), whereas cardiomyocyte-restricted deletion is lethal at 20-40 days, Mattiotti and colleagues are generating conditional knockouts to test if endogenous Fstl1 is required during heart repair.

Organ regeneration can also involve the dedifferentiation of mature cells, as highlighted by Guo Huang (University of California, San Francisco, USA). Epicardial enhancers and the transcription factors that promote the reversion to epicardial progenitor cells have previously been mapped (Huang et al., 2012). A functional genomics screen is now underway to promote adult mouse cardiomyocyte proliferation, based on viral delivery of the top 50 genes that are downregulated more severely in cardiomyocytes than in epicardial cells postnatally. Indeed, Huang reported that, in long-term cultured adult cells, pooled viruses induce EdU labeling in more than 25% of the myocytes, with serial imaging confirming myocyte division. In a chemical biology approach (in collaboration with Ralph Marcucio, University of California, San Francisco, USA) to enhance regeneration in mice (see Choi et al., 2013), an unexpected but encouraging result is the regrowth of digits after their amputation for the purpose of identification.

Materials science and tissue engineering approaches to cardiovascular repair

Engineering myocardium *in vitro*, via the creation of tissue patches, was discussed by Nenad Bursac (Duke University, Durham, USA) (Zhang et al., 2013; Bian et al., 2014; Jackman et al., 2015). Embedding cardiomyocytes from human induced pluripotent stem cells (hiPSCs) in hydrogel, polymerized in a nylon frame, allowed the formation of 60 µm-thick tissue patches that exhibit features of maturation. These patches become vascularized spontaneously after grafting to mice and can be scaled up in size. A limitation that remains is the lack of electrical integration between patch and heart, potentially due to scarring and lack of myocyte migration. An intriguing approach to overcome this barrier is to engineer cardiac fibroblasts that bridge electrical conduction between patch and heart cardiomyocytes.

Molly Stevens (Imperial College London, UK) gave an overview of her approaches to enhance regenerative medicine through materials science, for example via the use of conductive polymers to influence arrhythmia inducibility, sutureless bioadhesion, and via the micropatterning of biomaterials to better mimic the anisotropy of the heart. Nano-analytical electron microscopy is also being applied to gain insights into cardiovascular tissue calcification (Bertazzo et al., 2013; Autefage et al., 2015), while porous silicon nanoneedles have been devised for sensing intracellular pH changes and enzyme activities, and for delivering drugs, nanoparticles and nucleic acids (Chiappini et al., 2015a,b).

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Filippo Perbellini (Imperial College London, UK) discussed cardiac tissue engineering from a physiologist's perspective. When using pluripotent cell derivatives, chamber identity must be matched to the electrophysiological properties of the host milieu. However, essential features such as action potential morphology, calcium extrusion and calcium content in sarcoplasmic reticulum are remarkably plastic in culture, reflecting experimental variables rather than being innate (Du et al., 2015). Given this plasticity, cell-substrate interactions have been studied in efforts to promote maturity, and several strategies, including changes to biochemical composition, stiffness and topographical micropatterning, have been used. These can promote the calcium cycling properties of myocytes so that they better resemble those of adult myocytes (Rao et al., 2013; Trantidou et al., 2014). Myocardial slices are also being used to provide templates that are even more organotypic in nature (Camelliti et al., 2011).

Reprogramming and genome editing

Eric Olson (University of Texas Southwestern Medical Center, Dallas, USA) – the scientific father, grandfather, or godfather of many of the workshop participants – described high-throughput studies to enhance the efficiency of cardiac reprogramming. Identified in a screen of 192 human protein kinases, AKT markedly increases the yield of beating cardiomyocytes generated by the combination of *GATA4*, *HAND2*, *MEF2C* and *TBX5*; this was attributed to the AKT target MTOR, which improves bioenergetics, and to AKT downregulating FOXO3A, a transcription factor that inhibits reprogramming (Zhou et al., 2015). Wider screens encompassing transcription factors, epigenetic regulators, nuclear receptors and cytokines yielded eight novel inducers as well as novel barriers. Olson also discussed how genome editing by CRISPR-Cas9 has been applied to prevent Duchenne muscular dystrophy in *mdx* mice, which have a truncated form of dystrophin (Long et al., 2014). Translating this to humans, Olson and colleagues devised a gene-editing strategy that exploited non-homologous end-joining-mediated permanent exon skipping and the high redundancy of dystrophin's internal rod domains. Proof-of-concept results demonstrate that the defect can be corrected in hiPSC-derived cardiomyocytes (hiPSC-CMs) from a patient with muscular dystrophy. Following this, adeno-associated viruses were engineered for delivering Cas9 and guide RNAs, and were used to rescue dystrophin protein in *mdx* mice to functionally significant degrees (Long et al., 2016).

Kathy Ivey (Gladstone Institute of Cardiovascular Disease, San Francisco, USA) also spoke on hiPSC gene editing using CRISPR-Cas9. Building on information obtained from model organisms on transcriptional circuits controlling cardiogenesis, Gladstone investigators launched the BioFulcrum initiative (https://gladstone.org/our-science/biofulcrum), aimed at defining transcription factor localization and network dynamics during human cardiomyocyte differentiation. To achieve this, in differentiating hiPSCs, ∼25 endogenous transcription factor genes are being epitope tagged, enabling studies of factor binding throughout the genome (via ChIP-Seq) and of protein-protein complexes (via affinity-purification mass spectrometry). Concomitantly, fluorescent reporter iPSC lines are being engineered, indicative respectively of the atrial (*SLN*) or ventricular (*MYL3*) myocyte lineage.

The use of reprogrammed cells to model human disorders was discussed by Deepak Srivastava (Gladstone Institute of Cardiovascular Disease, San Francisco, USA), focusing on *GATA4* mutations that cause congenital heart disease (Garg et al., 2003). Patients harboring a G296S mutation develop cardiomyopathy as teenagers despite repair of the septal and valve defects in childhood. Using hiPSC-CMs derived from these patients, the Srivastava lab is using bioengineering approaches to study how this mutation affects myocyte alignment, electrophysiology and mechanical performance. Further studies, using ChIP-Seq for GATA4 and TBX5, are providing insights into how the *GATA4* mutation disrupts interactions with TBX5 at 'super-enhancers' and modulates the cardiogenic gene program. Srivastava also discussed his group's efforts to enhance direct programming to cardiomyocytes using small-molecule screening, carried out in collaboration with Gladstone investigator Sheng Ding (Wang et al., 2014; Zhu et al., 2015). Using a transcription factor cocktail comprising Gata4, Mef2c and Tbx5 as starting point, the group identified molecules that could improve the quality and accelerate the reprogramming process *in vitro* and *in vivo*. Building on this, an all-chemical cocktail of nine compounds has been successfully devised for the cardiogenic conversion of human fibroblasts.

Obstacles to direct cardiac reprogramming were discussed by Li Qian (University of North Carolina, Chapel Hill, USA). To address relative dosing and overcome the heterogeneity of viral transduction when factors are delivered singly, an optimized polycistronic vector was developed for *Mef2c*, *Gata4* and *Tbx5* (Ma et al., 2015; Wang et al., 2015a,b). Qian also investigated epigenetic impediments and, using a loss-of-function screen, showed that loss of the Polycomb repressive complex 1 component Bmi1 enhances cardiac programming 3-fold, which was generalizable to various reported gene cocktails and recipient somatic cell types. Augmentation did not involve Bmi1 target genes with known roles in cell proliferation [*p16INK4a* and *p19ARF* (both are transcripts encoded by the *Cdkn2a* locus), *p53* (*Trp53*)], but rather an unrecognized function of the protein that involves direct binding to the promoters of cardiogenic genes (*Gata4*, *Nkx2-5*, *Isl1*, *Pitx2*, *Tbx20*) and the exchange of repressive histone marks for active ones. Accordingly, *Bmi1*-depleted cells could be reprogrammed successfully using just Mef2c and Tbx5.

Alessandra Moretti (Technical University Munich, Germany) used an iPSC-based approach to investigate the pathology of the hereditary heart muscle disorder arrhythmogenic right ventricular dysplasia (ARVC). Cardiomyocytes generated from iPSCs derived from ARVC patients spontaneously acquire brown-beige fat characteristics and progressively upregulate adipocytic genes while exhibiting disruption of sarcomere organization and loss of myocytic identity. The ability of cardiomyocytes to spontaneously adopt a beige adipocyte phenotype in disease suggests a possible developmental relationship between these two cell lineages. Using genetic fate mapping in the mouse and the clonal differentiation of embryonic stem cell-derived cardiovascular precursors, Moretti identified a subset of primordial $Isl1^{+}/Wt1^{+}$ progenitors that are bipotent and give rise to both cardiomyocyte and adipocyte lineages. These findings illustrate a naturally occurring conversion of heart muscle into beige fat in human disease and provide a foundation to prevent or revert the process as a potential therapeutic strategy.

Together, these reprograming and genome-editing approaches demonstrate the ever-increasing sophistication of methods to impose a cardiomyocyte phenotype in non-muscle cells, the utility of patient-specific stem cell-derived cardiomyocytes as a springboard to translation, and the dawn of CRISPR-Cas9 methods for targeted mutagenesis or gene correction.

Transcription factor networks

Benoit Bruneau (Gladstone Institute of Cardiovascular Disease, San Francisco, USA) provided a perspective on gene regulatory

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networks and chromatin remodeling, focusing on *Tbx5* and *Nkx2-5*. Tbx5, Nkx2-5 and Gata4 binding sites were analyzed by ChIP-exo for ultra-high resolution mapping (Rhee and Pugh, 2011; Luna-Zurita et al., 2016). Sites of triple, double or single occupancy were identified, the triple sites correlating with active chromatin and differential expression. Factor knockouts result in ectopic binding, e.g. Tbx5 to *Pecam1* in the absence of Nkx2-5, and Nkx2-5 to *Corin* in the absence of Tbx5, concordant with the upregulation of endocardial and neural genes. The factors together possess 10-fold higher binding affinity than either alone, and by crystallography the ternary complex encompassing both was seen to induce DNA bending. Thus, heterotypic factor interactions contribute not only to decreased binding but also to ectopic binding.

The binding of Nkx2-5 to embryonic cardiac enhancers was also investigated by Laurent Dupays (Crick Institute, London, UK). ChIP on E11.5 mouse heart identified more than 2500 Nkx2-5 binding sites (Blow et al., 2010; Dupays et al., 2015). Two binding motifs were identified, one of which was reminiscent of that for Meis1, a cardiac cell cycle regulator (Stankunas et al., 2008; Mahmoud et al., 2013). Meis1 and Nkx2-5 are expressed successively in cardiac progenitors and bind sequentially to this motif. At E9.5, Meis1 enrichment predominates in the anterior heart field, while Nkx2-5 predominates in the heart, resembling the factors' respective levels of expression. The 'hits' for Nkx2-5 alone were principally cardiac, whereas dually bound sites associated at least equally with diverse other functions. Notably, only half of the target genes bound by Nkx2-5 are downregulated in mice defective for *Nkx2-5*; the other half are upregulated, indicating that repression is an important direct role of this factor.

Brian Black (University of California, San Francisco, USA) discussed transcriptional regulation of *Prkaa2*, which encodes the alpha2 subunit of AMP-activated protein kinase (AMPK), a key therapeutic target in metabolic syndrome, type 2 diabetes and cardioprotection (Qi and Young, 2015). Epigenetic datasets were mined for evolutionary conservation of non-coding sequences within 100 kb of the *Prkaa2* start site, and a predicted cardiac enhancer was identified. A 1 kb region identified in these screens was tested *in vivo* in transgenic mouse embryos and was found to direct *lacZ* expression exclusively to the heart throughout embryogenesis and in the adult. Conversely, CRISPR-mediated deletion of the region substantially reduced endogenous *Prkaa2* expression, establishing that this element serves as a bona fide enhancer. Deletion analyses in *lacZ* transgenic mice revealed a necessary and sufficient segment for enhancer activity *in vivo*. The transcriptional regulation of the enhancer by an upstream transcriptional network was discussed, and a cooperative mechanism for this enhancer and for cardiac enhancers in general was proposed.

Transcriptomic, proteomic and genomic studies

Several presentations analyzed the more global changes occurring during heart regeneration. For example, building on the regenerative capacity of the neonatal mouse (Porrello et al., 2011, 2013), Enzo Porrello (University of Queensland, Brisbane, Australia) described transcriptomic profiling of adult versus neonatal infarction, surveying diverse participating cells including cardiomyocytes, endothelial cells, fibroblasts and leukocytes. By principal component analysis, Porrello showed that leukocytes and fibroblasts revert to a neonatal state after infarction; myocytes and endothelial cells, by contrast, fail to revert to a neonatal state, even though myocytes account for many differentially expressed genes. To revert an adult myocyte to a neonatal state might thus require overcoming epigenetic barriers that are laid down in postnatal development. Although *in vitro* models of engineered human heart tissue would be attractive for the study of this problem, most suffer from immaturity, laboriousness, and lack of amenability to high-throughput screening. For these reasons, 96-well plates with flexible micropillars in each well – enabling a more native architecture and semi-automated analysis of force production – have been devised and applied successfully to screen for combinatorial conditions that promote cardiomyocyte maturation.

Studies mapping the transcriptome of the injured mouse heart were presented by Rob Janssen (Academic Medical Center, Amsterdam, The Netherlands), who suggested that regeneration is initiated but not completed by adult cardiomyocytes in the infarct border zone. *Nppa* and *Nppb* are highly expressed in the border zone in mice and fish, with antibodies to pSmad1/5/8 suggesting that the BMP pathway is activated there. Spatially resolved expression profiles after coronary artery ligation are currently being analyzed by genome-wide RNA tomography (Tomo-Seq) (Junker et al., 2014), as implemented for the regenerating zebrafish heart (Wu et al., 2016), while an *Nppb*-driven far-red fluorescent protein, Katushka, is being used to label the border zone following infarction (Sergeeva et al., 2014) and will enable the comparison of positive and negative myocytes.

Antonio Beltrami (University of Udine, Italy) examined the gene expression profiles of cardiac-derived multipotent stem cells, which have unique features, including a mesothelial signature (Verardo et al., 2014), and coexpress mesenchymal and pericytic markers together with c-Kit (Avolio et al., 2015). In immunodeficient mice subjected to infarction, a combination of human cardiac stem cells plus a pericyte/mesenchymal population from human saphenous veins (Campagnolo et al., 2010) was superior to each individually in promoting myocyte proliferation and c-Kit$^+$ stem cell recruitment plus promoting angiogenesis, suggesting a rationale for combination therapy (Avolio et al., 2015).

Finally, Nadia Rosenthal (The Jackson Laboratory, Bar Harbor, USA) investigated subtle complexities in immune cells and fibroblasts during tissue repair. Characteristically, wound healing entails infiltration by neutrophils, then macrophages, then T cells and mast cells, with macrophages shifting from a pro-inflammatory (M1) to anti-inflammatory (M2) state. Insulin-like growth factor 1 (IGF1) has been implicated in multiple macrophage responses, and Rosenthal showed that its deletion just in the macrophage compartment abrogates M2 polarization and muscle regrowth in mice. Conversely, overexpressing IGF1 in skeletal muscle accelerates the M2 phase and regeneration (Tonkin et al., 2015), as does transgenic overexpression in the heart or clinically relevant delivery to adult myocardium with adeno-associated virus AAV9 (Gallego-Colon et al., 2015, 2016). Fibroblast action is also important in determining infarct outcome: cardiac fibroblasts express an unexpected cardiogenic gene program, and infarct repair is improved by ablating *Tbx20* in the fibroblast compartment (Furtado et al., 2014, 2016). Cardiac fibroblast number is also markedly reduced in IGF1-overexpressing transgenic mice, and in more effectively regenerating strains, as revealed by comparing ∼30 strains from the Collaborative Cross recombinant inbred mouse panel (Churchill et al., 2004; Threadgill and Churchill, 2012); this approach has already defined several infarct-related quantitative trait loci.

Perspectives

A closing summary by James Martin highlighted the meeting's principal recurring and emerging themes and was followed by an engaging discussion of why the human heart appears to be so

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limited in its regenerative capacity. Participants agreed that restarting cardiomyocyte proliferation, either via Hippo, Neuregulin or newly discovered pathways, is incontrovertibly feasible, but how might one best exploit those targets for investigation and therapy? Studies of zebrafish heart repair now prompt the mining of regeneration-specific enhancers, genetic programs, and cellular factors that promote myocyte expansion. The identity and function of other cell types that contribute to heart repair remain puzzling but are beginning to be unraveled via single-cell analyses, imaging and transcriptomics. Still other secrets of restorative growth have succumbed to innovations in advanced mouse genetics and genome editing, while input from the field of material science and bioengineering has provided the means to enhance reprogramming, durable engraftment and myocyte maturity. The prospects for translation to human hearts are so imminent that several leading zebrafish and mouse labs have already embarked on large-mammal studies, building solidly on the discoveries made in accessible, genetically tractable reductionist model systems.

Acknowledgements

I am grateful to Brian Black and James Martin for organizing this extraordinary meeting, and The Company of Biologists for transcendent logistical support and hospitality. This workshop was made singular by vigorous contributions from all the participants, who are warmly thanked for their many helpful comments on this text.

Competing interests

The author declares no competing or financial interests.

Funding

Work in the author's laboratory is supported by the British Heart Foundation [CH/08/002, RE/13/4/30184, RG/15/1/31165, RM/13/1/30157] and the Wellcome Trust [WT10638].

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