Development 134, 1631-1633 (2007) doi:10.1242/dev.002014

The heart's Da Vinci code: a renaissance at Keystone

Benoit G. Bruneau[1,*] and Brian L. Black[2]

At a recent Keystone symposium on 'Molecular Pathways in Cardiac Development and Disease' in Colorado, significant advances in the understanding of heart development were discussed. The identification and isolation of cardiovascular progenitors, their modulation by secreted factors, and some tantalizing insights into cardiac regeneration were some of the highlights of what was characterized by some as a renaissance in cardiovascular development.

Introduction

When cardiovascular biologists recently gathered in Breckenridge, Colorado for the Keystone Symposium on 'Molecular Pathways in Cardiac Development and Disease' (organized by Kenneth Chien, Eric Olson and Ketty Schwartz), it was clear that significant change had taken place in the field. Kenneth Chien (Harvard Medical School, Boston, MA, USA) put it best when he declared in his opening comments that we were witnessing a renaissance in cardiovascular biology. Indeed, if the era of descriptive anatomy was our medieval times, then, with the findings discussed at this conference, we are certainly entering the age of enlightenment. What profound discoveries are ushering in this new age? Largely, it is the much-anticipated and late-coming identification of cardiovascular precursors, and the understanding of their differentiation and allocation to different segments of the forming cardiovascular system. Greatly related to this is the understanding of how these precursors can be identified and expanded ex vivo, which has finally brought some hope towards the long-sought goals of cardiac regeneration. From a developmental biologist's perspective, this also has provided vindication for those who professed that, to regenerate a tissue, one must understand how it develops in the first place!

Progenitors netted

The identification and harnessing of cardiovascular lineages was one of the big topics of discussion. Until recently, it was assumed that the heart arises from a crescent of differentiating mesoderm cells that somehow expand to form the nascent heart (e.g. Srivastava and Olson, 2000). This dogma came under serious scrutiny in a series of papers that showed that the origin of a large portion of the developing heart lay outside of the cardiac crescent (reviewed by Buckingham et al., 2005). This idea seemed almost heretical at the time, but shortly thereafter it received crucial support with the molecular identification of one of the controlling forces behind this 'second heart field' (SHF), a transcription factor named Isl1 (Cai et al., 2003). Indeed, *Isl1* is expressed in the SHF, and lineage-tracing experiments showed that the descendants of *Isl1*-expressing cells populate most of the heart, except for the free wall of the left ventricle. Furthermore, mice lacking *Isl1* fail to develop a right ventricle or outflow tract, which are largely derived from the SHF. More recently, it has been shown that *Isl1*-expressing precursor cells persist in late fetal and perinatal life, suggesting that they might function as myoblast equivalents, poised to regenerate a diseased or damaged heart (Laugwitz et al., 2005).

At the Breckenridge conference, Chien started things off by taking these findings a significant step forward. His team identified, based on the expression of *Isl1*, an early multipotential embryonic cardiovascular precursor cell that could differentiate into cardiac myocytes, smooth muscle cells and endothelial cells (Moretti et al., 2006) (Fig. 1). These progenitors could be isolated from mouse embryos, as well as from embryonic stem (ES) cells differentiated into embryoid bodies. Importantly, clonal analysis showed that a single Isl1-positive cell could give rise to all three cardiovascular lineages. They then further defined the cardiac lineage as being positive for Isl1, for the transcription factor Nkx2-5 and for the receptor Flk1 (also known as Kdr – Mouse Genome Informatics). This echoed another paper that was published last year, which also identified Flk1+ multipotent cardiovascular progenitors (Kattman et al., 2006), although contrasted with a third paper that identified early bipotential precursors as an Nkx2-5+/Sca-1+ population (Wu et al., 2006). Thus, the concepts and tools of hematopoietic developmental biology, namely multipotent lineage progenitors and cell-sorting technologies, appear to have been successfully adopted by cardiovascular biologists, and we now finally have a handle on the developmental origin and the differentiation sequence of multiple cardiovascular lineages. Brian Black (UCSF, San Francisco, CA, USA) presented results of experiments aimed at understanding the molecular control of the lineage transition from SHF progenitors to cardiac myocytes, in the context of the developing organism. It was known that an enhancer from the *Mef2c* gene controls its own expression specifically in the anterior portion of the SHF (known as the anterior heart field, or AHF), and that this transcriptional regulation is dependent on Isl1-binding sites (Dodou et al., 2004). A puzzling aspect of these findings is the apparent lack of overlap between *Mef2c* expression (which includes the AHF and also its derivatives) and *Isl1* expression (which includes the SHF but not its derivatives). How could the Isl1-binding sites regulate the expression of *Mef2c* beyond the domain of Isl1 expression? It turns out that Isl1 binding may effect epigenetic changes at the chromatin level, presumably allowing the persistent binding of subsequent transcriptional regulators, such as Gata4 and, probably, others. The theme of chromatin remodeling was further explored by Benoit Bruneau (Gladstone Institute of Cardiovascular Disease, San Francisco, CA, USA.), who showed that the de novo activation of cardiac genes by cardiac DNA-binding transcription factors, such as Tbx5 and Nkx2-5, largely depends on the recruitment of chromatin-remodeling complexes.

Owen Prall (Richard Harvey's group, Victor Chang Cardiac Research Institute, Sydney, Australia) presented intriguing findings that showed a complex role for Nkx2-5 in regulating the balance between the specification and proliferation of cardiac progenitor cells in the heart fields, and their allocation to the forming heart. He showed that, in mice lacking *Nkx2-5*, there is at first an overproduction of progenitor cells, followed, paradoxically, by a proliferative collapse in SHF cells and a reduced deployment of SHF cells to the outflow tract and right ventricle. Both of these effects were attributable to the loss of an Nkx2-5-dependent negative-

[1]Gladstone Institute of Cardiovascular Disease and Department of Pediatrics and [2]Cardiovascular Research Institute and Department of Biochemistry, University of California, San Francisco, CA 94158, USA.

*Author for correspondence (e-mail: bbruneau@gladstone.ucsf.edu)

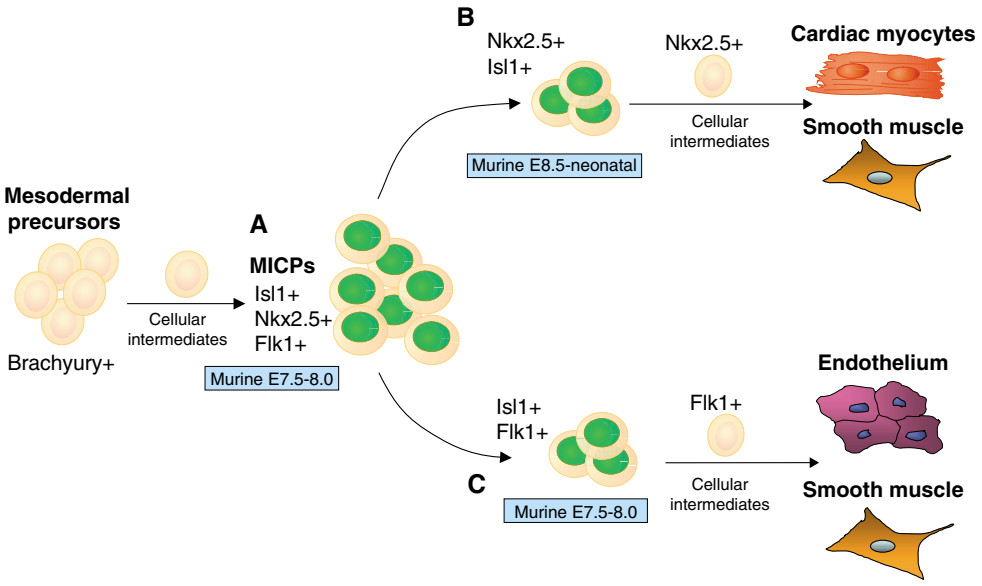

Fig.1. The cellular hierarchy of cardiac progenitors and their lineage specification. (**A**) Multipotent *Isl1*+ cardiovascular progenitor cells (MICPs) express *Isl1*, *Nkx2.5* and *Flk1*, and can give rise to all three lineages – cardiac muscle, smooth muscle and endothelium – during embryonic heart development. (**B**) Loss of *Flk1* expression yields *Isl1*+/*Nkx2.5*+ cells that are similar to postnatal *Isl1*+ progenitors and that can generate cardiac or smooth-muscle lineages. (**C**) *Isl1*+/*Flk1*+ cells are more restricted in their ability to differentiate; they can give rise to either endothelial or smooth-muscle cells. Adapted from Moretti et al. (Moretti et al., 2006), with permission.

feedback circuit that targets bone morphogenetic protein (BMP) gene expression (Prall et al., 2007). These findings were extended to show that this mechanism is likely to be relevant to human congenital heart defects, which can be caused by *NKX2-5* mutations.

A Wnt turnaround: promoting cardiac differentiation

Another major focus of the meeting was on the signals that are important for the differentiation of cardiovascular precursors into their final state. Particular attention was paid to the differentiation of precursors into cardiac myocytes. A surprising consensus to emerge at the meeting was that the secreted Wnt family of signaling molecules are crucial positive modulators of cardiac differentiation. This was rather surprising because, once again, it was going against 'firmly established' dogma; evidence from frog- and chicken-embryo manipulation, and from the culture of P19 embryonal carcinoma cells, have all converged towards one conclusion: that Wnts signaling via the canonical β-catenin-dependent pathway are negative regulators of cardiac differentiation (Marvin et al., 2001; Pandur et al., 2002; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). However convincing, these findings are at odds with the positive role of Wnt signaling in *Drosophila* cardiogenesis (Park et al., 1996; Wu et al., 1995).

At the Keystone meeting in Breckenridge, several investigators presented evidence that Wnts, in fact, can act as positive regulators of cardiogenesis via the canonical pathway. Deepak Srivastava (Gladstone Institute of Cardiovascular Disease, San Francisco, CA, USA) and Michael Schneider (Baylor College of Medicine, Houston, TX, USA) showed that Wnts, or the activation of the Wnt signaling pathway, could promote cardiogenesis in cultured ES cells that are allowed to differentiate into embryoid bodies. These findings have gained support from a recent publication that describes a positive and negative timing-dependent role for Wnts in cardiogenesis from ES cells (Naito et al., 2006). Thus, Wnts may play a dual role in cardiac development by acting as positive regulators at first and then switching to a negative-regulatory role subsequently. Interestingly, Schneider presented evidence that seemed to demonstrate that the pro-cardiogenic action of Wnts in embryoid bodies that have been derived from ES cells is, in fact, partly due to cell non-autonomous effects of Wnts, perhaps via the induction of Sox17 and Sox17-dependent genes in adjacent endodermal cells (Liu et al., 2007). However, other studies performed in vivo have indicated that Wnts might be acting in this context in a cell-autonomous manner. As discussed by Srivastava and Edward Morrisey (University of Pennsylvania, Philadelphia, PA, USA), the deletion of β-catenin in cardiac precursors results in the loss of cardiac structures, whereas the activation of β-catenin in the same cells leads to the formation of ectopic cardiac tissue. In vivo, Srivastava reported, β-catenin appears to function not in cell specification or migration, but rather in the expansion of the SHF progenitors, consistent with findings in ES cells.

Regenerating the heart?

The discussions about progenitors and their regulation by extrinsic factors prompted thoughts of cardiac regeneration. However, very little evidence shows that this might be possible. One exciting recent development has been the identification of the mechanisms by which the zebrafish heart can regenerate. Although previous work had shown that, unlike in mammalian hearts, this regeneration was possible (Poss et al., 2002), the mechanisms underlying this phenomenon remained rather mysterious. Kenneth Poss (Duke University, Durham, NC, USA) presented recent evidence that resident cardiovascular precursors can be recruited to the site of injury to repopulate the missing tissue, and that a recapitulation of the developmental stages of cardiogenesis takes place during this process (Lepilina et al., 2006). Furthermore, he demonstrated that the initial step in the response to injury is a reprogramming of the entire epicardium, followed by epithelial-mesenchymal transformation of these cells and subsequent neovascularization (Lepilina et al., 2006). The location and identity of dormant regenerative precursors remains mysterious, although Poss presented new data that suggests that cellular and molecular mechanisms important for cardiac regeneration also help to mediate the growth and homeostatic maintenance of the adult heart. Whether similar events can be forced upon mammalian myocardium remains to be seen. However, promising results were presented by Paul Riley (UCL Institute of Child Health, London, UK), who showed that Thymosin β4, an actin-binding protein, is essential for the formation of the coronary vasculature from the epicardium, and that exogenous Thymosin β4 could induce the recruitment of adult epicardial cells

to stimulate neovascularization (Smart et al., 2007). These findings provide an exciting potential means by which to help an injured or ischemic myocardium to repair, as had been suggested from previous findings that have shown that Thymosin β4 has a beneficial effect on infarcted hearts in mice (Bock-Marquette et al., 2004). These results also indicate a mechanism by which injured myocardium could be revascularized, which is perhaps an important step in facilitating regeneration.

Transcriptional and post-transcriptional regulation

Significant advances in our understanding of the transcriptional and post-transcriptional regulation of heart development and maturation were also presented at this meeting. Important new insights into the transcriptional control of the development of the cardiac conduction system – the specialized cells that propagate cardiac impulses – were presented by Ivan Moskowitz (University of Chicago, Chicago, IL, USA), who described the identification of the helix-loop-helix factor Id2 as an essential regulator of the patterning and function of the cardiac conduction system. Id2 was identified as lying downstream of Tbx5 and Nkx2-5, a pair of transcription factors that have previously been shown to influence arrhythmias in humans and mice when haploinsufficient (Jay et al., 2004; Mori and Bruneau, 2004; Moskowitz et al., 2004; Schott et al., 1998). Indeed, Moskowitz showed that compound haploinsufficiency of *Tbx5* and *Nkx2-5* appears to completely abrogate ventricular conduction-system specification.

Post-transcriptional regulation has finally entered its age of enlightenment with regard to heart development, where microRNAs and their regulatory mechanisms have been painted onto the cardiac canvas. Srivastava presented work on the loss-of-function of a muscle-restricted microRNA, *miR-1-2* (also known as *Mirn1-2* – Mouse Genome Informatics), which has previously been implicated in heart development (Zhao et al., 2005). Mice lacking *miR-1-2* have a range of phenotypes, including: cell cycle dysregulation, which results in the hyper-proliferation of cardiac myocytes; structural defects that include ventricular septal defects; and postnatal electrophysiological defects. This range of defects reflects the probable role of microRNAs in modulating multiple targets. The essential role of microRNAs in cardiac development was also strongly reinforced by Eric Olson (UT Southwestern, Dallas, TX, USA), who also presented exciting new data concerning microRNA-regulated events in the post-natal maintenance of the heart, including the altered expression of several microRNA genes in stressed hearts and their role in the pathological response of the heart to hypertrophy (van Rooij et al., 2006).

Conclusion

The molecular picture of the heart, and how this organ develops and functions, is far from being fully painted; but, if the initial brush strokes from our current renaissance in cardiac biology are anything to go by, then our interpretation of this vital organ, once sketched so elegantly by da Vinci himself in 1510, will truly be worth comparing to the works of the great Leonardo himself.

We thank D. Srivastava for critical reading of the manuscript, and all of the presenters cited for comments and clarifications.

References

Bock-Marquette, I., Saxena, A., White, M. D., Dimaio, J. M. and Srivastava, D. (2004). Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature* **432**, 466-472.

Buckingham, M., Meilhac, S. and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nat. Rev. Genet.* **6**, 826-835.

Cai, C. L., Liang, X., Shi, Y., Chu, P. H., Pfaff, S. L., Chen, J. and Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev. Cell* **5**, 877-889.

Dodou, E., Verzi, M. P., Anderson, J. P., Xu, S. M. and Black, B. L. (2004). Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development* **131**, 3931-3942.

Jay, P. Y., Harris, B. S., Maguire, C. T., Buerger, A., Wakimoto, H., Tanaka, M., Kupershmidt, S., Roden, D. M., Schultheiss, T. M., O'Brien, T. X. et al. (2004). Nkx2-5 mutation causes anatomic hypoplasia of the cardiac conduction system. *J. Clin. Invest.* **113**, 1130-1137.

Kattman, S. J., Huber, T. L. and Keller, G. M. (2006). Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev. Cell* **11**, 723-732.

Laugwitz, K. L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L. Z., Cai, C. L., Lu, M. M., Reth, M. et al. (2005). Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-653.

Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G. and Poss, K. D. (2006). A dynamic epicardial injury response supports progenitor cell activity during ebrafish heart regeneration. *Cell* **127**, 607-619.

Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R. J. and Schneider, M. D. (2007). Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **104**, 3859-3864.

Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M. and Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev.* **15**, 316-327.

Moretti, A., Caron, L., Nakano, A., Lam, J. T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S. et al. (2006). Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127**, 1151-1165.

Mori, A. D. and Bruneau, B. G. (2004). TBX5 mutations and congenital heart disease: Holt-Oram syndrome revealed. *Curr. Opin. Cardiol.* **19**, 211-215.

Moskowitz, I. P. G., Pizard, A., Patel, V. V., Bruneau, B. G., Kim, J. B., Kupershmidt, S., Roden, D., Berul, C. I., Seidman, C. E. and Seidman, J. G. (2004). The T-Box transcription factor TBX5 is required for the patterning and maturation ofthe murine cardiac conduction system. *Development* **131**, 4107-4116.

Naito, A. T., Shiojima, I., Akazawa, H., Hidaka, K., Morisaki, T., Kikuchi, A. and Komuro, I. (2006). Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc. Natl. Acad. Sci. USA* **103**, 19812-19817.

Pandur, P., Lasche, M., Eisenberg, L. M. and Kuhl, M. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* **418**, 636-641.

Park, M., Wu, X., Golden, K., Axelrod, J. D. and Bodmer, R. (1996). The wingless signaling pathway is directly involved in Drosophila heart development. *Dev. Biol.* **177**, 104-116.

Poss, K. D., Wilson, L. G. and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* **298**, 2188-2190.

Prall, O. W., Menon, M. K., Sollaway, M. J., Watanabe, Y., Zaffran, S., Bajolle, F., Biben, C., McBride, J. J., Robertson, B. R., Chaulet, H. et al. (2007). An Nkx2-5/Bmp2/Smad1 negative feedback loop controls second heart field progenitor specification and proliferation. *Cell* **128**, 947-959.

Schneider, V. A. and Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. *Genes Dev.* **15**, 304-315.

Schott, J.-J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B., Seidman, C. E. and Seidman, J. G. (1998). Congenital heart disease caused by mutations in the transcription factor *NKX2-5*. *Science* **281**, 108-111.

Smart, N., Risebro, C. A., Melville, A. A., Moses, K., Schwartz, R. J., Chien, K. R. and Riley, P. R. (2007). Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature* **445**, 177-182.

Srivastava, D. and Olson, E. N. (2000). A genetic blueprint for cardiac development. *Nature* **407**, 221-226.

Tzahor, E. and Lassar, A. B. (2001). Wnt signals from the neural tube block ectopic cardiogenesis. *Genes Dev.* **15**, 255-260.

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A. and Olson, E. N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. USA* **103**, 18255-18260.

Wu, S. M., Fujiwara, Y., Cibulsky, S. M., Clapham, D. E., Lien, C. L., Schultheiss, T. M. and Orkin, S. H. (2006). Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127**, 1137-1150.

Wu, X., Golden, K. and Bodmer, R. (1995). Heart development in Drosophila requires the segment polarity gene wingless. *Dev. Biol.* **169**, 619-628.

Zhao, Y., Samal, E. and Srivastava, D. (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* **436**, 214-220.