“String Theory” of c-kit<sup>pos</sup> Cardiac Cells
A New Paradigm Regarding the Nature of These Cells That May Reconcile Apparently Discrepant Results

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Abstract: Although numerous preclinical investigations have consistently demonstrated salubrious effects of c-kit<sup>pos</sup> cardiac cells administered after myocardial infarction, the mechanism of action remains highly controversial. We and others have found little or no evidence that these cells differentiate into mature functional cardiomyocytes, suggesting paracrine effects. In this review, we propose a new paradigm predicated on a comprehensive analysis of the literature, including studies of cardiac development; we have (facetiously) dubbed this conceptual construct “string theory” of c-kit<sup>pos</sup> cardiac cells because it reconciles multifarious and sometimes apparently discrepant results. There is strong evidence that, during development, the c-kit receptor is expressed in different pools of cardiac progenitors (some capable of robust cardiomyogenesis and others with little or no contribution to myocytes). According, c-kit positivity, in itself, does not define the embryonic origins, lineage capabilities, or differentiation capacities of specific cardiac progenitors. C-kit<sup>pos</sup> cells derived from the first heart field exhibit cardiomyogenic potential during development, but these cells are likely depleted shortly before or after birth. The residual c-kit<sup>pos</sup> cells found in the adult heart are probably of proepicardial origin, possess a mesenchymal phenotype (resembling bone marrow mesenchymal stem/stromal cells), and are capable of contributing significantly only to nonmyocytic lineages (fibroblasts, smooth muscle cells, and endothelial cells). If these 2 populations (first heart field and proepicardium) express different levels of c-kit, the cardiomyogenic potential of first heart field progenitors might be reconciled with recent results of c-kit<sup>pos</sup> cell lineage tracing studies. The concept that c-kit expression in the adult heart identifies epicardium-derived, noncardiomyogenic precursors with a mesenchymal phenotype helps to explain the beneficial effects of c-kit<sup>pos</sup> cell administration to ischemically damaged hearts despite the observed paucity of cardiomyogenic differentiation of these cells. (Circ Res. 2015;116:1216-1230. DOI: 10.1161/CIRCRESAHA.116.305557.)

Key Words: muscle development ▪ myocytes, cardiac ▪ regeneration

Because of the encouraging results of both preclinical<sup>1–5</sup> and clinical<sup>6</sup> studies, c-kit<sup>pos</sup>/cluster of differentiation (CD) 45<sup>neg</sup>/hematopoietic lineage (lin)<sup>neg</sup> cardiac cells (herewith referred to as c-kit<sup>pos</sup> cardiac cells) have emerged as one of the most attractive cell types for therapeutic application. At the preclinical level, numerous investigations conducted by many independent laboratories in a wide variety of animal models of ischemic cardiomyopathy have consistently documented salubrious effects of exogenous c-kit<sup>pos</sup> cardiac cells on left ventricular function and structure, including regeneration of dead myocardium.<sup>1–5</sup> At the clinical level, a small phase I study (the Cardiac Stem cells In Patients with Ischaemic cardiomyopathy [SCIPIO] trial) has documented the safety of autologous c-kit<sup>pos</sup> cardiac cell administration in patients with ischemic heart failure.<sup>6</sup> Although SCIPIO was not designed to assess efficacy, its results suggest that c-kit<sup>pos</sup> cardiac cells may impart beneficial effects on left ventricular function, quality of life, functional class, and infarct size,<sup>6</sup> thus providing a rationale for larger trials aimed at determining efficacy.

Despite these promising results, however, there continues to be outspoken skepticism regarding the use of c-kit<sup>pos</sup> cardiac cells as therapeutic agents.<sup>7,8</sup> We believe that an important factor fueling this skepticism is the inadequate evidence that either endogenous or exogenous adult c-kit<sup>pos</sup> cardiac cells differentiate into a relevant number of mature functional myocytes. Here we offer a new paradigm aimed at reconciling discrepant results obtained by different laboratories with respect to the therapeutic utility and differentiation potential of c-kit<sup>pos</sup> cardiac cells. Our conceptual construct is predicated on a comprehensive review of a large amount of work published by many independent groups over the past 2 decades. We believe that the theorem expounded herein provides a unifying theory that incorporates opposing, but perhaps not mutually exclusive,
positions regarding the direct contributions of c-kitpos cardiac cells to cardiomyogenesis.

The Controversy

In 2003, Beltrami et al.10 reported the discovery, in a rodent model, of resident c-kitpos/flinpos cardiac cells that were able to give rise to all cardiac lineages including cardiomyocytes. Over the past decade, however, conflicting results have been obtained with respect to the cardiomyogenic ability of c-kitpos cardiac cells. Although some in vitro studies have suggested that these cells express stemness-associated markers and early cardiac markers such as octamer-binding transcription factor 4 (Oct4), NK2 transcription factor related, locus 5 (Nkx2.5), and GATA binding factor 4 (GATA4), among others, and some sarcomeric proteins,3,10,11 formation of mature cardiomyocytes has not been observed12–14; furthermore, the artificial in vitro conditions used in those studies may promote a pattern of protein expression that is not likely to occur in vivo.13,14 Indeed, in the in vivo setting, reports of adult cardiomyocyte formation10,15,16 have not been reproduced by several laboratories including our own.1,5,11,12,17–22 We1,5,21 and others11,12,22 have found that c-kitpos cardiac cells transplanted in infarcted hearts do not differentiate into mature myocytes to a significant extent, implying that paracrine mechanisms must be responsible for the functional improvement.1,5,5,17,22 Efforts to elucidate the multifaceted paracrine mechanisms of c-kitpos cells, as well as other cell types, are currently underway.23,24

Whether the aforementioned lack of maturation is due to intrinsic inability of cells to differentiate into mature cardiomyocytes, extremely poor survival and engraftment, or compromised differentiation potential caused by suboptimal in vitro expansion remains to be established. It is possible that when they are removed from the heart and expanded in vitro, these cells partially lose their differentiation potential because of an impairment of complex in vivo cell signaling cascades that are essential for signaling cells to start proliferating and for eliciting targeted lineage commitment and differentiation. However, consistent with our observations with exogenous cells,1,2,4,5 recent work by the Molkentin group has also shed doubt on the cardiomyogenic nature of endogenous c-kitpos cardiac cells, suggesting instead a largely vasculogenic and adventitial lineage predisposition.16 In part, the discrepant results regarding the in vivo cardiogenic ability of exogenous c-kitpos cells1,5,10,15,17,19–21,25 might reflect differences in culture, isolation, or expansion conditions; however, in the van Berlo study,18 this was not an issue as the lineage-traced c-kitpos cells were of endogenous origin. Regardless of its causes, the failure of transplanted postnatal c-kitpos cardiac cells to assume a cardiac phenotype in most studies is a major limitation of cell therapy, which mandates a reassessment of the nature of these cells and commands a closer examination of their origins and natural innate functions, in an effort to ascertain (and possibly maximize) their potential for cardiogenic differentiation.

To this end, prior studies of fetal cardiac progenitors responsible for cardiomyogenesis and previous lineage tracing experiments in in vivo models may help evaluate the position of the c-kitpos cardiac population(s) within the known hierarchy of cardiac progenitors. This body of knowledge provides insights into the lineage commitment capabilities of c-kitpos cardiac cells and their likely predisposition toward mature phenotypes of the contractile, vascular, or adventitial compartments.

Discovery and Ancestry of c-kitpos Cardiac Cells

The initial discovery of c-kitpos cardiac cells was based on the fact that the c-kit receptor is expressed in hematopoietic progenitors;10 it was postulated that the presence of c-kit may identify an intramyocardial population of cardiac progenitors similar to that of the hematopoietic compartment. In fact, this is what Beltrami et al.10 found. They observed colocalization of c-kit with Nkx2.5, GATA-4, and Ki-67 but not with mature sarcomeric proteins, suggesting a precursor cell, ie, a proliferating cell that is apparently committed to cardiac lineage but lacks a mature phenotype. The absence of the hematopoietic markers CD34 and CD45 indicated that the cells were not immediately from the bone marrow. Therefore, it was concluded that the c-kitpos cardiac cells were derived from the embryonic cardiac compartments that ultimately give rise to the adult myocardium.10 Notably, this study did not address whether a pool of intracardiac cells expressing a c-kitpos phenotype represents a population of progenitors persisting in a quiescent state as remnants from embryonic development or whether c-kitpos cells arise de novo from c-kitneg cells resident within postnatal myocardium or even from c-kitneg cells in vitro.

Because the c-kit receptor (whose ligand is stem cell factor) plays an important role in prosurvival and proliferative signaling, it is possible that the c-kitpos phenotype may represent an intermediate progenitor, derived from an upstream c-kitneg, more undifferentiated cardiac progenitor in which c-kit expression increases in conjunction with cell cycle entry and differentiation. Beltrami et al.10 alluded to this possible hierarchy in their report of c-kitpos cardiac cells, which were found to largely coexpress Nkx2.5. This postulated upstream resident progenitor(s), however, has yet to be conclusively identified in the heart. Evidence of a similar phenotypic progression, now widely accepted, was observed in the bone marrow with the isolation in 2003 of c-kitneg hematopoietic stem cells, which were found to give rise to c-kitpos intermediate phenotypes that ultimately were able to reconstitute all mature hematopoietic lineages.26
So, what is the embryonic ancestry of c-kit<sup>+</sup> cardiac cells? Answering this question is important in order to ascertain their regenerative capacity, i.e., their ability to replace lost/damaged cardiac cells of various lineages. Clues to the position of c-kit<sup>+</sup> cells within the hierarchy of established cardiovascular phenotypes may be gleaned by examining their resident locations within the myocardium, the coexpression of known phenotypic, lineage-identifying transcription factors and cell surface markers in vivo and in vitro, and the results of contradictory lineage tracing studies such as those conducted by the Wu et al<sup>16</sup> and Molkentin laboratories.<sup>18</sup> Comparisons of these data with the established characteristics of known cardiac precursors should indicate a likely origin(s) of c-kit<sup>+</sup> cardiac cells, possible limitations of their differentiation capacity, and their relative contribution(s) to the adult heart.

**Mammalian Cardiac Developmental Biology**

The heart is the first functional organ formed during embryonic development, with cardiac progenitors specified in early gastrulation. Three spatially and temporally distinct cardiac precursors have been identified by lineage tracing experiments in embryonic development: cardiac mesodermal cells, proepicardial cells, and cardiac neural crest cells. These individual lineages have been established to give rise not only to specific cell types but also to regions of the mature heart.<sup>12,27,28</sup> Understanding the specification of these lineages in forming the mature heart is crucial if insights into the residual progenitors’ capacity to contribute to the contractile, vascular, and interstitial compartments, as well as response to injury, are to be gained. A brief synopsis of embryonic cardiac development is provided below (Figure 1).

Within the primitive streak, time-dependent differential coexpression of vascular endothelial growth factor receptor 2 (VEGFR2) (also known as kinase insert domain receptor [KDR] and fetal liver kinase 1 [Flk1]) allows the divergence of hematopoietic and peripheral vasculature progenitors from the cardiovascular progenitors that give rise to the heart and central portions of the great vessels.<sup>12,27,29–32</sup> The latter are designated by upregulation of the T-box transcription factors eomesoderm (Eomes) and mesoderm posterior 1 (Mesp1). These Mesp1+/Eomes+/KDR+ progenitors give rise to cardiac mesodermal cells that create the first and second heart fields (FHF and SHF, respectively) with thin endocardium and the proepicardium.<sup>12,27,29–34</sup> Cooperatively, these mesodermal progenitors and their progeny form the near entirety of the adult heart. The ectodermal originating cardiac neural crest cells also contribute to fetal cardiomyogenesis, but their contributions to the contractile compartment are thought to be minimal and, therefore, are not covered in this review.<sup>27,35,36</sup>

FHF progenitors in the cardiac crescent are exposed to local cytokines and growth factors, which induce differentiation and upregulation of essential cardiac regulators such as Nkx2.5, T-box transcription factor (Tbx)-5, and GATA4, among others. These transcription factors induce commitment to myocyte lineage and sarcomeric protein expression.<sup>12,27,29,30</sup> Progenitor tracking and lineage tracing studies have shown that the progeny of the FHF eventually gives rise to the myocytes and some smooth muscle cells that predominantly make up the left ventricular and both atria.<sup>12,16,27,33–35,37</sup> The endocardium may also arise from FHF progenitors as early simultaneous development is observed to form the primitive heart tube, although efforts are ongoing to further delineate early divergence of these 2 fields from one or more upstream progenitors.<sup>16,27,29,38,39</sup> Subsequent to FHF commitment and formation of the primitive heart tube, the SHF progenitors, identified by the expression of islet-1 transcription factor (Is11), Nkx2.5, and KDR, begin to proliferate and migrate, undergoing commitment and differentiation under the influence of local fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and Wnt signaling.<sup>12,27,30,40,41</sup> SHF progenitors have been shown to generate myocytes, some smooth muscle, and some endothelial constituents of the right ventricle and ventricular outflow tract.<sup>12,27,29,32,33,37,42–44</sup> Importantly, Is11+ progenitors have been found to lack c-kit and Sca-1<sup>12,40,41</sup> thus likely excluding this compartment as a source of residual myogenic progenitors having a c-kit<sup>+</sup> phenotype.

At this stage of cardiac development, the myocardium of the FHF and SHF, possessing only a thin endocardial lining within the contorting primitive heart tube,<sup>38</sup> is essentially naked, lacking adventitia, perforating vasculature, or surrounding epicardium. These constituents have been traced to arise from distinct proepicardial progenitor populations that express the transcription factors Wilms’ tumor protein (Wt1) and Tbx-18,<sup>12,27,28,35,43,45–48</sup> largely giving rise to adventitial and smooth muscle lineages, as well as Scleraxis (Scx) and Semaphorin3D (Sema3D), giving rise to adventitia and some vascular endothelium not of endocardial origin.<sup>49</sup> Some of these proepicardial progenitors have been found within endocardial cushions, areas well known to be formed by early endocardial progenitors. This colocalization indicates that these 2 fields undergo intermigration, essentially cooperating to form the mature structures of the AV valves and cardiac septa through epithelial to mesenchymal transition (EMT).<sup>50</sup> It is currently unclear whether these proepicardial populations stem from Is11+/Nkx2.5+ precursors of the SHF or are separately derived lineages. Tracing studies show that these progenitors migrate over the surface of the exposed myocardium, derived from the FHF and SHF, and form the epicardium and epicardium-derived cells (EPDCs).<sup>12,45,47,50–53</sup> Once formation of the epicardium is complete, epicardial cells proliferate in a direction parallel to the basement membrane, resulting in thickening of the epicardial lining, or perpendicular to the basement membrane, undergoing EMT beginning around E12.5 to 13.5. Ultimately, penetrating mesenchymally transitioned EPDCs, which populate the subepicardial region, migrate inward to form the coronary plexus (which later becomes the coronary vasculature, with contributions of endocardium-derived endothelial cells<sup>54–56</sup>) and cardiac adventitial fibroblasts. Additionally, the epicardium and EPDCs are involved in septation and function to stimulate myocardial growth and myocyte division,<sup>12,27,28,51,53,57</sup> specifically to aid formation of compact myocardium. Endocardium-derived adventitia aids in forming the inner trabecular myocardium.<sup>58</sup> A detailed hierarchy of the aforementioned fetal cardiac progenitor phenotypes is illustrated in Figure 1.
Figure 1. Proposed position of c-kit<sup>pos</sup> intermediates in the hierarchy of cardiac progenitors in the fetal heart. A and B. Arising first during cardiac development from a common pericardia mesodermal progenitor (brachyury T [Bry]+/mesoderm posterior 1 [Mesp1]+/omesodermin [Eomes]+/kinase insert domain receptor [KDR]+), endocardial (Bry+/KDR+/NK2 transcription factor related, locus 5 [Nkx2.5]+/islet-1 transcription factor [Isl-1]+) and first heart field (FHF; Bry+/Nkx2.5+/KDR−/c-kit<sup>pos</sup>) progenitors diverge early; the latter have been shown to have dedicated cardiomyocyte and smooth muscle cell bipotential differentiation capacity and to include c-kit<sup>pos</sup> intermediates. The expression of c-kit is postulated to be low because although these progenitors have been found to be c-kit<sup>pos</sup>, the van Berlo study failed to demonstrate their lineage contribution to the cardiomyocyte compartment in the adult heart, and it has been proposed that recombination in the van Berlo model is less effective in the presence of low levels of c-kit. Endocardial progenitors have not been shown to express c-kit. C, Subsequently arising SHF progenitors have not been shown to express c-kit. C, Subsequently arising SHF progenitors ([Isl-1+/Nkx2.5+/c-kit<sup>pos</sup>]) are c-kit negative and contribute to cardiomyocytes, smooth muscle cells, and vascular endothelium. D, Proepicardial progenitors (with diverging Wt1+/Tbx18+ and Sema3D/Scx+ populations) arise later from a mesodermal or SHF progenitor, giving rise to vasculogenic lineages and nearly all of cardiac adventitia including fibroblasts (lineages identified as having partially come from c-kit<sup>pos</sup> cells in the van Berlo study). Proepicardial progenitors expressing WT1 and Tbx18 undergo epithelial-to-mesenchymal transition (EMT) characterized by an upregulation of c-kit, resulting in c-kit<sup>pos</sup> intermediate phenotypes. The same is likely true for Sema3D/Scx expressing progenitors. The expression of c-kit in these proepicardial progenitors is postulated to be high because, in the van Berlo study, it was sufficient to induce recombination in adventitial lineages (which do not arise for FHF or SHF progenitors) and vascular lineages, in contrast to that of known c-kit<sup>pos</sup> FHF progenitors, which remained unlabeled. The hierarchy illustrated herein shows that c-kit expression is not limited to one cardiac progenitor and does not in itself define one specific cardiac precursor population. Shown at the bottom of the figure are the relative contributions of epicardium-derived cells to each cardiac lineage in fetal development according to the evidence outlined above. MSCs indicate mesenchymal stromal/stem cells.
It has recently been suggested that EPDCs may generate cardiomyocytes in fetal development, but this is currently unresolved. Questions have been raised regarding the specificity of the initial model that used Tbx-18 for in vivo tracing of EPDCs. However, similar subsequent evaluation of EPDCs by Zhou et al using Wilm’s tumor protein (WT1) also suggested that EPDCs can in fact contribute to mature cardiomyocytes during fetal cardiogenesis although this was rare. The same group also performed tracing studies of WT1+ epicardial cells in adult mice but did not find that these cells contribute to cardiomyocytes or endothelium after infarction; lineage commitment after ischemic injury-induced epicardial activation was primarily limited to smooth muscle and adventitial cells. Importantly, the study did observe that epicardial activation did occur as a result of ischemic injury, leading to proliferation and migration of EPDCs into the damaged myocardium in a reparative role. However, the aforementioned findings would support the concept that the differentiation capacity of WT1+ epicardial cells that persists into adulthood is less than that present in fetal development, because a more limited lineage commitment, restricted almost entirely to non-myocytes, was seen in adult mice. Scx/Sema3D+ cells were found to be a distinct population of proepicardial cells having only 33% overlapping coexpression of either WT1 or Tbx-18. Scx/Sema3D+ cells were found to give rise predominantly to coronary endothelial cells and adventitial cells with some additional contributions to smooth muscle, and rarely cardiomyocytes in the embryonic heart. This disproportionally low magnitude of cardiomyogenic potential mirrors that observed by the Zhou et al tracing study of WT1+ cells. Although initial studies in zebrafish suggested that activation of epicardial progenitors was responsible for cardiomyocyte replacement after injury, more recent work has shown that they act by inducing division of existing cardiomyocytes; epicardial cells were traced to give rise only to nonmyocyte lineages in that model. The current consensus is that the direct contribution of EPDCs to the myocardium is minimal and that cardiomyocyte differentiation is a rarity among EPDCs, at least in the postnatal heart. A progenitor hierarchy of adult EPDCs, with proposed phenotypic intermediates, is illustrated in Figure 2.

Recent studies of the origin of the endocardium, its formation, and its eventual contribution to mature cardiac lineages have found that its proportional contributions to mature lineages is similar to that attributed to proepicardium-derived cells. The endocardium arises very early in cardiac embryogenesis, simultaneously with the FHF, likely stemming from a common progenitor. Endocardial cells have been shown to arise from brachyury T (Bry) / fetal liver kinase 1 (Flk1/KDR) / Nkx2.5+ progenitors forming the primitive heart tube. These progenitors are distinct from hemangioblast precursors and are identified by a distinct expression profile (an E-cadherinlow, Flk1low, nuclear factor of activated T-cells, cytoplasmic 1 [NF-ATc1] + phenotype). NF-ATc1 was found to be expressed exclusively in endocardium, providing a lineage specific marker that enables differentiation of the endocardium from other endothelial cell types. Tracing and knockout studies performed by de la Pompa et al demonstrated that endocardial cells not only contribute to a subset of cardiac endothelial cells but also are integral to cardiac cushion formation, valvulogenesis, septation of the atria, ventricles, and aortopulmonary trunks, as well as to guiding myocardial trabeculation. These processes are governed by EMT of endocardial cells (similar with respect to mechanism and signaling pathways to that widely recognized to occur in EPDCs) that precipitates differential commitment to various mature cardiac lineages. The complex regulatory pathways underlying EMT of endocardial cells (as well as that of EPDCs) involve Notch, transforming growth factor-β superfamilies, small mothers against decapentaplegic (SMAD) proteins, Wnt/β-catenin, and bone morphogenetic proteins (BMP) signaling among others. Comprehensive reviews of these signaling cascades have recently been published, NF-ATc1 null mice, which lacked endocardium and therefore endocardial contributions to cardiac morphogenesis, showed marked abnormalities in trunkal, valvular, and septal formation, which were ultimately embryonically lethal. Interestingly, myocardial, adventitial, and most vascular endothelial compartments were found to be unaffected, indicating that the endocardium does not contribute significantly to these compartments. Similarly, studies in Tie-1/TEK(Tie2) null mice showed early embryonic lethality with impairment not only of endocardium formation but also of valvular and septal derivatives, and a lack of myocardial trabeculation. Interestingly, there was no impairment of early cardiomyocyte formation. It remains unclear, however, whether there are subpopulations of endocardial cells not defined by NF-ATc1 or Tie1/TEK expression that may contribute to these lineages.

**Placing c-kitpos Cells Within the Developmental Hierarchy of Cardiac Progenitor Phenotypes**

As supposed residual progenitors remaining from embryonic development, c-kitpos cardiac cells should be able to be attributed to derivation from one of these aforementioned precursors; if so, this would provide insights into their predisposition to form the various mature cardiac phenotypes. Clues to this assignment can be gained from available data on the location and phenotype of c-kitpos cells and from lineage tracing studies. In the aggregate, these data, detailed below, support the concept that c-kitpos cardiac cells likely represent intermediate phenotypes from more than one progenitor compartment within embryonic cardiomyogenesis, and that c-kit expression, in itself, does not define one specific cardiac progenitor. Indeed, c-kit expression has been found in intermediate phenotypes in very early bipotential myogenic/FHF progenitors as well as in epicardium-derived cells that undergo EMT to largely make vascular and adventitial lineages. The same may be true of c-kitpos cells isolated from endocardial biopsies (this will be discussed later). C-kit expression in these various progenitor lineages in the developing heart may vary not only temporally and spatially but also in the absolute levels of protein expressed. We suggest that these factors may account for discrepant results obtained by many groups in characterizing c-kitpos cells. We provide below a critical appraisal of the literature in an attempt to reconcile these differences.
Evidence for c-kit Expression in Early FHF Progenitors

As mentioned above, the FHF progenitors give rise exclusively to cardiomyocytes and smooth muscle cells.\textsuperscript{12,33–35,37} It has been shown that the simultaneously developing FHF progenitors and endocardium, although possibly originating from a common upstream mesodermal precursor cell, diverge very early with discrete specification to respective nonoverlapping lineages.\textsuperscript{16,35,37–39,54} Direct evidence supporting a c-kit\textsuperscript{pos} intermediate phenotype of FHF progenitor cells was provided in a seminal paper by Wu et al in 2006.\textsuperscript{16} In this work, the authors utilized both in vitro studies of embryonic stem cells and in vivo Nkx2.5-enhanced green fluorescent protein (eGFP) transgenic mice to examine the lineage specification of Nkx2.5\textsuperscript{+} cardiac progenitors throughout embryonic cardiomyogenesis. They found that, in vitro, cardiac differentiation of embryonic stem cells produced a subpopulation of Nkx2.5\textsuperscript{+}/c-kit\textsuperscript{pos} progenitors, lacking Flk1/Tie2(TEK) expression, which exhibited specific bipotential differentiation capacity toward cardiomyocytes and smooth muscle cells.\textsuperscript{16} However, Nkx2.5\textsuperscript{+}/c-kit\textsuperscript{neg} cells showed higher ability to directly differentiate into cardiomyocytes and smooth muscle cells in vitro than did Nkx2.5\textsuperscript{+}/c-kit\textsuperscript{pos} cells; therefore, c-kit positivity was viewed to be dispensable for cardiomyogenesis. Once isolated from E9.5 mouse hearts, Nkx2.5\textsuperscript{+}/c-kit\textsuperscript{pos} cells were able to form...
mature smooth muscle cells and cardiomyocytes. Thus, Nkx2.5+/c-kit<sup>pos</sup> cells at E9.5 showed similar dedicated bi-potential commitment to cardiomyocyte and smooth muscle lineages as did those from in vitro studies of embryonic stem cells and adoptive transfer studies in chick embryos. Evidence of c-kit expression in FHF progenitors is also provided by a study by Ferreira-Martins et al, in which c-kit<sup>pos</sup> cells were directly visualized in murine embryonic hearts at embryonic gestational day 6.5, a period of development currently thought to be confined solely to FHF progenitors during primitive heart tube formation, before the appearance of the SHF or the proepicardium. In summary, the study by Wu et al demonstrates that a subset of Nkx2.5+/eGFP+ cells coexpress c-kit in both in vitro and in vivo and that the Nkx2.5+/eGFP+/c-kit<sup>pos</sup> cells were able to generate smooth muscle cells as well as cardiomyocytes in single cell cloning. Interestingly, these cells were dedicated solely to these 2 lineages, specifically showing only bipotential differentiation capacity. Nkx2.5+/c-kit<sup>pos</sup> cells showed no overlapping expression of Flk1 or Tie2(TEK), indicating a lack of endothelial commitment, and no endothelial cells were observed to be generated from differentiation of these early Nkx2.5+/eGFP+/c-kit<sup>pos</sup> progenitors in vitro. This myogenic lineage restriction is consistent with that of FHF progenitors. These results would appear to be in conflict with the differentiation potential of c-kit<sup>pos</sup> cardiac cells observed by Ferreira-Martins et al, who found formation of not only cardiomyocytes and smooth muscle cells but also endothelial cells. However, Ferreira-Martins et al isolated c-kit<sup>pos</sup> cells much later in cardiac development (E16-18), a time when FHF, SHF, and proepicardial development are all simultaneously taking place. Accordingly, the c-kit<sup>pos</sup> cardiac cell population utilized in that study may have been heterogeneous, with c-kit<sup>pos</sup> cells originating from multiple compartments, which would have resulted in a broader differentiation potential compared with that observed by Wu et al. Further analyses by Wu et al comparing c-kit<sup>pos</sup> and c-kit<sup>neg</sup> Nkx2.5+ progenitors supported the concept that the c-kit<sup>pos</sup>/Nkx2.5+ state is an upstream intermediate progenitor phenotype, which, upon commitment to smooth muscle and/or cardiomyocyte lineages, loses c-kit<sup>pos</sup> positivity, retaining only Nkx2.5. Importantly, c-kit expression was observed to be downregulated, with very few c-kit<sup>pos</sup> cells detected in the fetal murine heart by E15.5 despite ongoing cardiac development; thus, further myocyte formation after E15.5 may be ascribable to c-kit<sup>neg</sup> progenitors such as those described by Wu et al (Nkx2.5+/c-kit<sup>neg</sup> cells) and/or to proliferation of cardiomyocytes themselves. In this connection, division of existing cardiomyocytes, rather than formation of new myocytes from pools of undifferentiated residual progenitors, appears to be the predominant mechanism for cardiomyogenesis in the neonatal heart, although this ability is lost within weeks of birth.

**Evidence That Cells Expressing c-kit Are of Proepicardial Origin and Mesenchymal in Nature**

Numerous independent laboratories have provided evidence supporting the concept that c-kit<sup>pos</sup> cardiac cells, especially in the postnatal heart, are derived from the proepicardium and are mesenchymal in nature (Table). This body of evidence can be summarized as follows.

**Location of Adult c-kit<sup>pos</sup> Cells**

C-kit<sup>pos</sup> cardiac cells in adult human and murine hearts inhabit predominantly the subepicardium and adjacent myocardial interstitium, regions derived from proepicardial progenitors. Immunohistochemical labeling of c-kit<sup>pos</sup> cells shows an epicardial to endocardial gradient.

**Expression of Proepicardial Markers in Some c-kit<sup>pos</sup> Cells**

Additional evidence for the proepicardial origin (and EMT) of these cells is provided by recent studies showing that many murine epicardial WT1 and Tbx18 expressing cells also coexpress c-kit and that this expression increases with epicardial activation.

**In Vitro Generation of c-kit<sup>pos</sup> Cells by EMT of Epicardial Cells**

Human c-kit<sup>pos</sup> cells can be generated in vitro by inducing EMT of human epicardial cells with transforming growth factor-β. In vitro–generated c-kit<sup>pos</sup> cells exhibit expression of mesenchymal markers at the mRNA level similar to that of c-kit<sup>pos</sup> cardiac cells analyzed directly after isolation from human cardiac tissue. This is in contrast to the expression profile of directly isolated epicardial mesenchymal cells. An important implication of these observations is that a c-kit<sup>pos</sup> phenotype can arise in vitro from c-kit<sup>neg</sup> cells, raising the possibility that c-kit<sup>pos</sup> cells isolated and expanded in vitro for therapeutic purposes may not represent, as commonly thought, a resident c-kit<sup>pos</sup> embryonic remnant within the myocardium.

**Expression of Mesenchymal Markers in c-kit<sup>pos</sup> Cells**

Many studies by independent groups have consistently shown that adult human c-kit<sup>pos</sup> cardiac cells express CD105, CD29, and other mesenchymal-associated markers both in vivo and in vitro. The in vivo expression, assessed by immunohistochemical staining, indicates that this mesenchymal phenotype is inherent to c-kit<sup>pos</sup> cardiac cells from adult humans and mice and is not the result of in vitro artifacts or culture drift. In the van Berlo study, small numbers of cardiomyocytes were found to originate from c-kit<sup>pos</sup> progenitors; at least some of these were ascribed to cellular fusion, a phenomenon that is known to occur in mesenchymal stromal/stem cells (MSCs).

**Differentiation Potential of c-kit<sup>pos</sup> Cells**

When placed in directed differentiation conditions, adult c-kit<sup>pos</sup> cells have shown a capacity to express markers of osteocytes, chondrocytes, and adipocytes typical of MSCs in addition to some mature cardiac proteins.

**C-kit Expression in MSCs**

MSC populations from various tissues (oral, adipose, bone marrow, and cardiac tissue) express c-kit, indicating that this protein is associated with mesenchymal lineages and that...
Table. Evidence That c-kit Is Expressed in More Than One Progenitor Compartment and That c-kit Expression in Itself Does Not Define a Capacity for Myogenic Differentiation Potential

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<th>Evidence for an early c-kit&lt;sup&gt;low&lt;/sup&gt; intermediate phenotype of FHF progenitors during development</th>
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<td><strong>Detection of c-kit&lt;sup&gt;pos&lt;/sup&gt; cardiac cells during embryonic cardiomyogenesis during a period confined to FHF progenitors</strong>&lt;sup&gt;35&lt;/sup&gt;: C-kit&lt;sup&gt;pos&lt;/sup&gt; cardiac cells were observed to arise at E6.5 in murine cardiomyogenesis, a time confined to FHF progenitor formation of the primitive heart tube, before the appearance of the SHF and proepicardium&lt;sup&gt;77,69&lt;/sup&gt;.</td>
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<td><strong>Coexpression of Nkx2.5 and c-kit in vitro in ESC-derived cardiac progenitors</strong>&lt;sup&gt;16&lt;/sup&gt;: Cardiac differentiation of ESCs in vitro produced a subpopulation of Nkx2.5+/c-kit&lt;sup&gt;pos&lt;/sup&gt; progenitors, lacking Flk-1/Tie2(Tek) expression, with specific bipotential differentiation capacity toward cardiomyocytes and smooth muscle cells. However, Nkx2.5+/c-kit&lt;sup&gt;−/−&lt;/sup&gt; cells showed greater ability to differentiate into cardiomyocytes and smooth muscle cells in vitro; therefore, c-kit positivity was viewed to be dispensable because it did not define a sole cardiomyogenic progenitor pool.</td>
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<td><strong>Isolation of E9.5 Nkx2.5+/c-kit&lt;sup&gt;pos&lt;/sup&gt; cells from murine hearts</strong>&lt;sup&gt;16&lt;/sup&gt;: Cells isolated from freshly isolated E9.5 mouse hearts exhibited similar dedicated bipotential commitment to cardiomyocyte and smooth muscle lineages as did in vitro ESC-derived studies and adoptive transfer studies in chick embryos.</td>
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<td><strong>Combination of the observations noted above with lack of recombined progeny in the van Berlo study</strong>&lt;sup&gt;16,18&lt;/sup&gt;: The van Berlo study failed to detect recombination events in FHF progenitors, indicating either that these progenitors do not express c-kit or that c-kit expression in these precursors is sufficiently low that it does not induce recombination. The latter scenario would appear to be most plausible in view of the evidence provided by Ferreira-Martins et al&lt;sup&gt;15&lt;/sup&gt; and Wu et al&lt;sup&gt;16&lt;/sup&gt;.</td>
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**Evidence for a c-kit<sup>high</sup> proepicardial intermediate progenitor with mesenchymal nature that persists into adulthood**

**Location**: C-kit<sup>pos</sup> cells inhabit regions of postnatal myocardium derived from proepicardial progenitors: epicardium, subepicardium, and adjacent interstitium of the outer myocardium, with an epicardial to endocardial gradient<sup>66-67</sup>.

**Expression of proepicardial markers**: Fetal and adult c-kit<sup>pos</sup> cells have been found to express the proepicardial transcription factors WT-1 and Tbx-18 in vivo and in vitro<sup>67,71</sup>.

**In vitro generation of c-kit<sup>pos</sup> cardiac cells**: C-kit<sup>pos</sup> cells have been generated in vitro by TGF-β-induced EMT of adult epicardial cells<sup>58</sup>.

**Coexpression of mesenchymal markers**: Adult human c-kit<sup>pos</sup> cardiac cells display a mesenchymal phenotype, with CD105 and CD29 positivity among other markers<sup>51,68,72-79</sup>.

**Differentiation capacity**: Adult human c-kit<sup>pos</sup> cells can express mesenchymal lineage markers of adipocytes, osteocytes, and chondrocytes on directed differentiation in vitro<sup>13,72,73,80</sup>.

**C-kit<sup>pos</sup> phenotype in mesenchymal cells from various tissues**: A c-kit<sup>pos</sup> phenotype is observed in mesenchymal cells from cardiac, bone marrow, dermis, oral, and adipose tissues<sup>72,81-86</sup>.

**Lineage tracing studies**: In vivo constitutive and inducible Cre-recombinant tracing studies have shown that c-kit<sup>pos</sup> cells contribute to endothelial cells (which also may arise from hemangioblast precursors) and some interstitial and stromal cells in the adult murine myocardium<sup>18</sup> which are known to arise exclusively from proepicardial progenitors and EPDCs<sup>12,27,30,37</sup>. (Whether this is also true for endocardial cells remains unclear but is probable.) The level of c-kit expression in this model was high enough to induce recombination<sup>18</sup> despite the proposed objections regarding the model’s insensitivity to low expressers of c-kit<sup>51</sup>.

**Paracrine mechanism of action**: Adult c-kit<sup>pos</sup> cells work primarily through paracrine mechanisms (they exhibit minimal differentiation into mature phenotypes), a characteristic intrinsic to the known supportive nature of EPDCs toward the underlying myocardium<sup>5,11,12,17,27,30,35,37,46,71</sup>.

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CD indicates cluster of differentiation; EPDC, epicardium-derived cell; ESC, embryonic stem cell; FHF, first heart field; Flk-1, fetal liver kinase 1; Nkx2.5, NK2 transcription factor related, locus 5; SHF, second heart field; Tbx, T-box transcription factor; and WT, Wilm’s tumor protein.
Lineage Tracing Studies

Recently, van Berlo et al.\(^\text{18}\) conducted a c-kit\(^{\text{pos}}\) lineage tracing study in mice utilizing permanent recombination to track all progeny of c-kit expressing cells throughout cardiac organogenesis as well as after injury. Mature phenotypes arising from c-kit\(^{\text{pos}}\) progenitors were found to be mostly endothelial cells (which also may have arisen from c-kit\(^{\text{neg}}\) hemangioblast precursors and not exclusively from cardiac progenitors) and few smooth muscle and stromal interstitial cells including fibroblasts, but rarely cardiomyocytes.\(^{18}\) Concerns have been raised regarding the efficiency of recombination and the effect of the loss of a c-kit allele in this study.\(^{18}\) However, even if one assumes that there was suboptimal recombination in low expressers of c-kit (which would result in underestimation of the contribution of c-kit\(^{\text{pos}}\) cells to adult cardiac lineages), this would not invalidate the findings of positive recombination events in higher c-kit expressers and the mature cardiac lineage contributions thereof. Indeed, no presumption of inaccurate recombination has been raised, nor was such off target recombination observed by the authors in the validation of their murine model.\(^{18}\) The lineage distribution reported by van Berlo et al.\(^{18}\) would imply that these supposed high expressers of c-kit (c-kit\(^{\text{high}}\) cells) are likely derived from the proepicardium because the FHF and SHF have not been shown to contribute to fibroblasts or interstitial cells\(^{12,27,28}\) and smooth muscle cells from the FHF share a common precursor with cardiomyocytes generated from that compartment.\(^{16}\) Lineage tracing studies of WT1+ and Tbx-18+ proepicardial progenitors in fetal cardiomyogenesis have shown similar degrees of distribution toward noncardiomyocyte phenotypes as well as only a small contribution to mature cardiomyocytes, mirroring the observations of van Berlo et al.\(^{18,45,46}\) Further implications of a possible insensitivity to lower expressers of c-kit in the heart (c-kit\(^{\text{low}}\) cardiac cells) are discussed later.

Paracrine Mechanism of Action of Adult c-kit\(^{\text{pos}}\) Cells

Although bone marrow–derived MSCs have beneficial effects in the setting of ischemic cardiomyopathy, differentiation of these cells into cardiomyocytes seems unlikely\(^{23,88,90,91}\), rather, MSCs are thought to work via paracrine actions.\(^{23,24}\) Similarly, we have found that c-kit\(^{\text{pos}}\) cardiac cells also appear to work via paracrine actions.\(^{3,5,17}\) Although c-kit\(^{\text{pos}}\) cells administered in animal models of ischemic cardiomyopathy have been reported to differentiate into phenotypically mature cardiomyocytes on tissue histopathologic examination,\(^{0,15,92}\) we\(^{3,5,17}\) and others\(^{11,19,20,22,72}\) have not observed this phenomenon. Tracing studies of eGFP-labeled c-kit\(^{\text{pos}}\) cells have shown very limited engraftment, with isolated, small eGFP+ cells displaying a disorganized pattern of staining for sarcomeric proteins or smooth muscle actin\(^{1,5,17,19,20}\), rarely, if ever, are mature cardiomyocytes observed that are derived from transplanted cells. Despite this, administration of in vitro expanded c-kit\(^{\text{pos}}\) cardiac cells has been reproducibly beneficial in preclinical and clinical studies of heart failure, implying a paracrine mechanism, eg, antifibrotic or antiapoptotic actions, or activation of endogenous precursors triggered by factors released from the transplanted cells.\(^{3}\) This postulated paracrine mechanism would be consistent with a proepicardial origin because throughout development proepicardium-derived cells are known to support the myocardium by secreting a variety of beneficial growth factors.\(^{12,27,30,35,47,71}\) The specific paracrine mediators responsible for these beneficial effects are the focus of active investigation, and likely involve a host of pathways including microparticles and microRNA-mediated effects as well as release of growth factors and cytokines such as stromal cell-derived factor 1, vascular endothelial growth factor, and many others. Regardless of the precise mechanism(s) involved, the limited ability of adult transplanted c-kit\(^{\text{pos}}\) cells to acquire a mature cardiomyocytic phenotype is also consistent with the limited ability of proepicardium-derived cells to differentiate into myocytes.\(^{12,27,30,35,45,46}\)

Some may point to results of in vitro differentiation of adult c-kit\(^{\text{pos}}\) cells, along with coexpression of factors such as GATA4 in vitro and in vivo, as evidence to the contrary. However, the expression of GATA4, like that of Nkx2.5, is not restricted to cardiomyocyte precursors nor is it indicative of specific cardiomyocyte commitment. GATA4 knockout studies in murine embryos have concluded that this factor is expressed in, and necessary for, formation of the proepicardium and its derivatives,\(^{93,94}\) which is again consistent with a proepicardial origin of c-kit\(^{\text{pos}}\) cardiac cells. The finding that cardiac troponin T is expressed after in vitro differentiation or in vivo transplantation of c-kit\(^{\text{pos}}\) cells has been construed as evidence of cardiomyocyte differentiation; however, smooth muscle cells may also express cardiac troponin T.\(^{16,95}\) These facts highlight the fundamental importance of using multiple markers and methodologies to document differentiation into a specific lineage and to define an undifferentiated starting population. In vitro differentiation conditions are highly artificial because they utilize nonphysiological stimuli that may cause cellular drift potentially not indicative of what occurs in vivo.\(^{13,14,77}\) Direct evidence supporting this concept is the observation by Miyamoto et al.\(^{96}\) that in vitro expanded c-kit\(^{\text{pos}}\) cardiac cells cultured in cardiac differentiation medium expressed not only some native cardiac markers but also markers typical of adipose and skeletal muscle lineages. Because cells expressing these markers are not present within normal myocardium, it may be concluded that this in vitro behavior deviates from any normal function or derivation of c-kit\(^{\text{pos}}\) cardiac cells in vivo, irrespective from which compartment (FHF, proepicardial, or other) they originate, and can be considered a culture artifact or drift. Such observations bring into question the validity of relying on cardiomyogenic differentiation in vitro as a true representation of in vivo capability (vide infra).

Although the evidence summarized above supports the notion that adult c-kit\(^{\text{pos}}\) cells may be of proepicardial origin and share a mesenchymal-like phenotype, expressing canonical MSC markers, these cells appear to differ in a tissue-specific manner from conventional MSCs; for example, they differ from MSCs isolated from the bone marrow both functionally and in their ability to express multilineage markers of differentiation in vitro.\(^{19,72,97,98}\)

C-kit\(^{\text{pos}}\) Cells From Human Endomyocardial Biopsies

One potential objection to the concept that c-kit\(^{\text{pos}}\) cells originate entirely from the FHF or are of proepicardial origin is that these
cells have been isolated from endomyocardial biopsies obtained from the right ventricular septum. Such observations are not necessarily in conflict with the postulated origin of c-kit+ cardiac cells from the FHF or the proepicardium because it is possible that c-kit expression is not limited only to EMT of epicardial cells but occurs more broadly as a part of EMTs. EMT is well recognized to occur in endocardial epithelial cells that contribute to various cardiac structures such as atrioventricular cushions, valves, and septa as well as to vascular endothelium and cardiac adventitia, a pattern similar to the lineage capabilities of EPDCs. In-depth reviews of these phenomena have been recently published. Thus, endocardial cells obtained from endomyocardial biopsies (EMBs) may undergo EMT in vitro with resultant upregulation of c-kit expression. This would parallel that which has been observed in vitro in epicardial mesothelial cells.

Besides the observations of increased c-kit expression in epicardial EMT-induced in vivo and in vitro by transforming growth factor-β, there is mounting evidence that similar c-kit expression occurs in extracardiac tissues undergoing EMT as well as in EMT leading to tumorigenesis. Studies of in vitro transforming growth factor-β-induced EMT in noncardiac epithelial cell lines have shown an increase in expression of c-kit and mesenchymal markers, essentially mirroring the results obtained with induction of EMT in human epicardial mesothelium. These observations would indicate that c-kit upregulation is biologically integral to the process of EMT itself, independent from the cell type of origin. If this hypothesis is correct, the expansion of c-kit+ cells from endomyocardial biopsies could be explained by EMT of endocardial cells in vitro.

Another potential explanation for the isolation of c-kit+ cells from endocardial septal biopsies relates to the intermigration and cooperative function of EPDCs and endocardial cells within the outflow tracts and adjacent AV cushions during cardiogenesis and/or as a part of septation. Cells from both the epicardial and the endocardial fields work in tandem to perform complex structural rearrangements to complete the formation of a mature 4-chambered heart. It is possible that the subendocardium and adjacent interstitial adventitia consist of cells with embryonic ancestral heterogeneity, being of endocardial and proepicardial origin.

A Unifying Theory of c-kit Expression in the Heart

Taken together, the evidence reviewed above supports the concepts that (1) c-kit expression in the myocardium is not limited to one progenitor but is a property of cells that originate from multiple pools of progenitors in the developing and postnatal heart (eg, FHF, proepicardium), and (2) c-kit expression in itself does not define the embryonic origins, lineage capabilities, or differentiation capacities of the various progenitors. C-kit+ cardiac cells from the FHF show marked cardiomyogenic and smooth muscle differentiation capacity early in fetal development. However, there is inconclusive evidence that c-kit+ cells from this FHF compartment persist in the postnatal heart into adulthood. More likely, any residual progenitors from this field would exhibit only an Nkx2.5+ state because Wu et al observed a drastic downregulation of c-kit expression in Nkx2.5+ cells, with c-kit becoming nearly undetectable in E15.5 murine hearts. This may indicate depletion of the Nkx2.5+/c-kit+ early intermediate phenotypes within the FHF progenitor pool. Any subsequent progenitor proliferation and contributions to the contractile compartment past E15.5 might be attributed to the more mature Nkx2.5+/c-kit+ progenitors observed and characterized by Wu et al as well as to cardiomyocytes and smooth muscle cells themselves, as mounting evidence suggests.

Because no markers specific to the FHF have yet been identified that would allow segregation of c-kit+ cardiac populations, it is difficult to know what proportion of these cells in the postnatal myocardium, if any, is a remnant from the FHF with primary cardiomyogenic potential versus c-kit+ cells stemming from other compartments such as the proepicardium whose contributions during cardiomyogenesis are overwhelmingly to noncardiomyocyte lineages. It may reasonably be postulated that the number of c-kit+ cardiac cells is proportional to the proliferative activity of their progenitors and that the largest fraction of c-kit+ cardiac cells remaining in the adult myocardium represents the compartments with the largest proliferative and regenerative reserve. According to this hypothesis, the lack of appreciable myocyte replacement in the contractile compartment, in contrast to the overwhelming plasticity and reserve of the vascular and adventitial compartments (which encompass the progeny of non-FHF progenitors), would indicate that the adult c-kit+ cardiac cells represent intermediate phenotypes of these residual nonmyocyte contributing progenitor pools or even intermediates of recently described transdifferentiating cell types undergoing EMT such as vascular endothelial cells.

So, then, how can studies such as those conducted by Wu et al and van Berlo et al, with opposite conclusions regarding the cardiomyogenic capacity of c-kit+ cardiac cells, be reconciled assuming that the findings of both may in fact be valid? As discussed above, one possibility is that, as some have proposed, the van Berlo model was not sensitive to recombination in cases of very low c-kit expression (c-kit+ cells) and therefore only traced the lineage contributions of higher c-kit expressers (c-kit+ cells). The van Berlo study clearly shows that a large portion of cardiac endothelial cells (which may in part be progeny of c-kit+ hemangioblasts and not dedicated cardiac progenitors), as well as some smooth muscle and adventitial cells, arise from a progenitor with a c-kit+ intermediate phenotype. Again, this mature lineage distribution is not inconsistent with a proepicardial and/or endocardial origin. Additionally, this c-kit+ progenitor, which has a sufficiently robust c-kit expression to induce recombination in the van Berlo model, does not give rise to an appreciable number of cardiomyocytes, thus leaving the contractile compartment as the progeny of other progenitors. Assuming the validity of the findings of Wu et al, who clearly demonstrated the bipotential differentiation capacity (cardiomyocytes and smooth muscle cells) of an Nkx2.5+/c-kit+ progenitor very early in embryonic cardiomyogenesis, and those of Ferreira-Martins et al, who observed c-kit+ cardiac cells at embryonic gestational day 6.5, both consistent with FHF progenitors, the differences between the studies could be explained if these FHF c-kit+ cells possess lower levels of c-kit compared with cells of proepicardial/
endocardial origin (c-kit\textsuperscript{high} cells) and if the expression of c-kit in these c-kit\textsuperscript{low} cells was insufficient to induce recombination and visualization in the van Berlo model. According to this hypothesis, the contributions of FHF c-kit\textsuperscript{low} progenitors to the adult myocardium would be underestimated, as some have proposed.\textsuperscript{56} By segregating c-kit\textsuperscript{low} cardiac progenitors into c-kit\textsuperscript{high} and c-kit\textsuperscript{low} expressers, this conceptual construct would reconcile the Wu et al\textsuperscript{18} and van Berlo et al\textsuperscript{18} studies and allow for both to be included under one unifying paradigm.

Whether these postulated FHF c-kit\textsuperscript{low} cardiac cells persist into adulthood or are depleted early in embryonic development, as would be suggested by Wu et al\textsuperscript{16} and by studies of neonatal cardiac regeneration,\textsuperscript{62} remains to be conclusively elucidated. The evidence examined in this review regarding the characteristics of adult c-kit\textsuperscript{pos} cardiac cells that have been isolated and expanded from adult human myocardial samples would indicate that these c-kit\textsuperscript{pos} cardiac progenitors are no longer present in adult hearts. It is much more likely that cells isolated from adult human cardiac specimens are c-kit\textsuperscript{high} cells, not only for the reasons outlined above but also because of the methodology of magnetic-activated cell sorting that is utilized to isolate cells for clinical or preclinical uses. Magnetic immunoselection preferentially selects the highest expressers and highest retainers of the immunomagnetic ferrous beads; accordingly, low expressers of an antigen of interest are very likely to pass through the selection column together with negatively selected cells. In view of this, and considering the entire body of evidence discussed in this article, we believe that the cells expanded in vitro from adult cardiac tissue are c-kit\textsuperscript{high} expressers of proepicardial origin.

The likely proepicardial origin and mesenchymal nature of adult c-kit\textsuperscript{pos} cells may explain their predisposition to form endothelium and some smooth muscle and adventitial cells, and their lack of robust cardiomyocyte differentiation, which is consistent with the recently published lineage tracing analysis.\textsuperscript{18} Additionally, the ability to form cardiomyocytes appears to differ significantly between neonatal and adult c-kit\textsuperscript{pos} cells,\textsuperscript{11,102-104} the former can form cardiomyocytes, albeit to a limited extent, whereas the latter either have lost this ability or do so at a minuscule rate. This difference mirrors the aforementioned differential cardiomyogenic capacity of EPDCs in fetal/neonatal and adult mouse hearts,\textsuperscript{34,66} again suggesting a proepicardial origin.

**Endogenous Versus Exogenous c-kit\textsuperscript{pos} Cells**

The evidence reviewed above pertains to c-kit\textsuperscript{pos} cells residing in the heart (endogenous cells). An important question is whether their properties can be extrapolated to c-kit\textsuperscript{pos} cells isolated, cultured, and expanded in vitro (exogenous cells). What effect do in vitro conditions and expansion have on the inherent differentiation capacity of these cells?

As previously mentioned, it is theoretically possible that in vitro conditions increase or shift the differentiation capacity of c-kit\textsuperscript{pos} cells from certain lineages to others, possibly by disinhibition, resulting in increased cardiomyocyte formation, whereas in the in vivo setting environmental signals, especially in the adult heart, may limit this phenomenon, even in response to injury. However, evidence exists that this may not be the case.\textsuperscript{11} As indicated above, data regarding exogenous (expanded) c-kit\textsuperscript{pos} cells are conflicting: while some studies have concluded that these cells undergo full cardiomyogenic differentiation in the recipient heart,\textsuperscript{10,15,52,56,17,21} and others\textsuperscript{11,12,19,20,22} have found that these cells do not assume a cardiomyocytic phenotype when transplanted in vivo. The reason(s) for these discrepancies is unknown. Cells generated in one laboratory cannot be assumed to be identical to those generated in another laboratory, as even subtle differences in culture conditions may bring about phenotypic changes in cultured cells. In any case, the important concept here is that the cardiomyogenic potential (as well as other properties) of exogenous c-kit\textsuperscript{pos} cells is likely different from that of endogenous c-kit\textsuperscript{pos} cells. The former have been expanded and cultured extensively in highly artificial conditions that almost certainly affect cellular functions and may favor a selection of the fastest replicating subsets of cells.

Indeed, considering the dramatic differences between culture and in vivo conditions, it would be surprising if many cell properties were not affected. An obvious example is the population doubling time of cultured c-kit\textsuperscript{pos} cells (typically, <30 hours) which is much shorter than that of endogenous cells in vivo. Another example, described above, is the aberrant expression of noncardiac proteins that has been reported in c-kit\textsuperscript{pos} cells cultured in differentiation media.\textsuperscript{72,96} There are likely many other differences, which are not unexpected when one considers the very artificial (and often arbitrary) culture conditions and the enormous differences between the environment to which c-kit\textsuperscript{pos} cells are exposed in vitro and in vivo. In our opinion, extrapolation from artificial (and largely arbitrary) culture conditions to the very complex environment in the intact organism, with its myriad of signaling stimuli and other modulating influences (most of which remain poorly understood or unknown), is not warranted. Conclusions predicated on studies of exogenous c-kit\textsuperscript{pos} cells should not be extrapolated to endogenous cells and vice versa.

**Conclusions**

In this essay, we have proposed a unifying theory that reconciles ostensibly discrepant results obtained in studies of c-kit\textsuperscript{pos} cardiac cells over the past 2 decades. We have (facetiously) dubbed this construct the “string theory” of c-kit\textsuperscript{pos} cardiac cells (in analogy to the theory that has been proposed to explain the physical universe)\textsuperscript{105} because it reconciles multifarious and sometimes apparently discrepant results. We have also cautioned against extrapolating studies of endogenous c-kit\textsuperscript{pos} cells to those of exogenous (expanded) c-kit\textsuperscript{pos} cells and vice versa.

To recapitulate, multiple lines of evidence support the concept that c-kit is expressed in more than one fetal cardiac progenitor pool (ie, both FHF and mesenchymally transitioning proepicardium and EPDCs), and that its expression does not define one specific myogenic precursor. C-kit expression within these pools may vary not only temporally and spatially throughout cardiac development but also in terms of absolute protein levels. The apparently conflicting results of studies of endogenous c-kit\textsuperscript{pos} cells could be explained by the existence of two populations of intermediate cardiac precursors, low and high c-kit expressers (c-kit\textsuperscript{pos} and c-kit\textsuperscript{high}). The former would be derived from the FHF, give rise to cardiomyocytes and smooth muscle cells, and are likely depleted during fetal
cardiomyogenesis, thus not persisting within the adult heart; if they persist, they would likely escape isolation by conventional magnetic-activated cell sorting. The latter would be derived from the proepicardium, display a mesenchymal phenotype, give rise to some adventitial cells (including fibroblasts), smooth muscle cells, and endothelial cells, and persist in the adult heart, with a continuous cycle of epicardial cells undergoing EMT and migrating inward into the myocardium, especially in response to injury.93–96.105,106 These are likely the c-kitpos cells that are isolated with magnetic-activated cell sorting from adult myocardium. Because of their postulated lower levels of c-kit expression, the former may not recombine efficiently in a Cre knockin model such as the van Berlo study,87 thus yielding an underestimation of the contributions of FHF c-kithigh progenitors to the contractile compartment (myocytes and smooth muscle) during fetal development.

This paradigm accounts both for the robust cardiomyogenic differentiation of c-kitpos intermediates reported by Wu et al16 during development and for the recently observed proliﬁcity of endogenous c-kitpos cells to differentiate more toward interstitial and vascular lineages and less toward contracting myocytes reported by van Berlo et al.18 Furthermore, it illuminates the apparent paradox regarding the mechanism of action of exogenous c-kitpos cells isolated from adult hearts. Because MSCs are known to work primarily via paracrine mechanisms,23,24 the recognition that exogenous postnatal c-kit+ cardiac cells resemble the phenotype of traditional MSCs provides insights into the consistent functional beneﬁts afforded by these cells despite the paucity of their cardiomyocytic differentiation, and helps to reconcile the recent report that endogenous c-kitpos cells contribute minimally to restoring the cardiomyocyte compartment in the adult heart18 with the remarkable therapeutic actions of exogenous c-kitpos cells.3

This paradigm does not exclude the possibility that an early c-kit− intermediate phenotype of FHF progenitors may give rise to large numbers of cardiomyocytes, as was observed by Wu et al.16 Although the data reviewed above indirectly support our theorem, the presence of two (or more) populations of cardiac cells expressing different levels of c-kit (c-kitlow and c-kithigh cardiac cells) is presently a conjecture and needs to be veriﬁed experimentally. Clearly, more work is needed to differentiate subsets of c-kit expressing cells on the basis of multiple markers and to deﬁne residual pools of preferentially cardiomyogenic c-kitpos cells in the adult myocardium if they are in fact still present. Currently, it appears that the c-kitlow cardiac cells able to be isolated and expanded from postnatal myocardium for therapeutic purposes are limited to those without any signiﬁcant cardiomyogenic capability and represent intermediates from compartments other than the FHF (ie, proepicardium). If the goal is to maximize formation of new myocytes, new therapeutic approaches utilizing these proepicardial/endocardial c-kitpos cardiac cells, such as reprogramming techniques, rather than simple in vitro expansion and administration, may be useful to increase cardiomyocyte differentiation, especially in cells harvested from adult hearts that may show even more restricted lineage capabilities than those in fetal or neonatal development.11

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Disclosures
None.

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"String Theory" of c-kit^{POS} Cardiac Cells: A New Paradigm Regarding the Nature of These Cells That May Reconcile Apparently Discrepant Results
Matthew C.L. Keith and Roberto Bolli

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The *Circulation Research* article by Keith and Bolli ("String Theory" of c-kit<sup>+</sup> Cardiac Cells: A New Paradigm Regarding the Nature of These Cells That May Reconcile Apparently Discrepant Results. *Circ Res*. 2015;116:1216-1230. doi: 10.1161/CIRCRESAHA.116.305557) states that van Berlo et al (2014) observed that large numbers of fibroblasts and adventitial cells, some smooth muscle and endothelial cells, and rare cardiomyocytes originated from c-kit positive progenitors. However, van Berlo et al reported that only occasional fibroblasts and adventitial cells derived from c-kit positive progenitors in their studies. Accordingly, the review has been corrected to indicate that van Berlo et al (2014) observed that large numbers of endothelial cells, with some smooth muscle cells and fibroblasts, and more rarely cardiomyocytes, originated from c-kit positive progenitors in their murine model.

The authors apologize for this error, and the error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/116/7/1216.full