Stem Cell–Derived Nodal-Like Cardiomyocytes as a Novel Pharmacologic Tool: Insights from Sinoatrial Node Development and Function

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Abstract—Since the first reports on the isolation and differentiation of stem cells, and in particular since the early success in driving these cells down a cardiac lineage, there has been interest in the potential of such preparations in cardiac regenerative therapy. Much of the focus of such research has been on improving mechanical function after myocardial infarction; however, electrophysiologic studies of these preparations have revealed a heterogeneous mix of action potential characteristics, including some described as “pacemaker” or “nodal-like,” which in turn led to interest in the therapeutic potential of these preparations in the treatment of rhythm disorders; several proof-of-concept studies have used these cells to create a biologic alternative to electronic pacemakers. Further, there are additional potential applications of a preparation of pacemaker cells derived from stem cells, for example, in high-throughput screens of new chronotropic agents. All such applications require reasonably efficient methods for selecting or enriching the “nodal-like” cells, however, which in turn depends on first defining what constitutes a nodal-like cell since not all pacemaking cells are necessarily of nodal lineage. This review discusses the current state of the field in terms of characterizing sinoatrial-like cardiomyocytes derived from embryonic and induced pluripotent stem cells, markers that might be appropriate based on the current knowledge of the gene program leading to sinoatrial node development, what functional characteristics might be expected and desired based on studies of the sinoatrial node, and recent efforts at enrichment and selection of nodal-like cells.

I. Introduction

Since the early 1990s, numerous studies have been conducted in which stem cells of various types (embryonic, adult mesenchymal, induced pluripotent) and from multiple species (mouse, human) have been manipulated in some way to produce a preparation of cardiac-like cells (Wobus et al., 1991; Kehat et al., 2001; Chow et al., 2013; Robertson et al., 2013). What is generally obtained, however, is a heterogeneous and immature population of cells with characteristics reminiscent of (but not necessarily...
identical to) cells from distinct adult cardiac regions, including ventricle, atrium, and sinoatrial node (SAN) (Maltsev et al., 1993; Mummery et al., 2003). The vast majority of research studies and review articles have focused on producing ventricular-like cells that might be suitable for repairing the working myocardium after myocardial infarction or heart failure.

More recently, investigators have begun addressing the question of whether the SAN subtype within this heterogeneous cell population can be preferentially generated or selected (Zhu et al., 2010; Hashem and Claycomb, 2013). If successful, such an approach would have multiple applications. First, if a relatively pure preparation of cells characteristic of the adult SAN could be produced, it would provide an ideal model system for exploring the ionic basis of normal and abnormal automaticity within the SAN. Although there has been extensive study of this point, controversy remains (see section II). Further, relatively little of the research is based on human tissue, and thus an appropriate cell preparation of human origin would be a valuable tool. Second, such a preparation might provide an ideal source with which to create a “biological pacemaker” to replace current electronic pacemakers in treating rhythm disorders. Proof-of-concept experiments with biologic pacemakers have demonstrated some success with the implantation of fetal (Ruhparwar et al., 2002) or adult (Zhang et al., 2011) SAN cells and beating embryoid bodies (EBs) derived from embryonic stem cells (ESCs) (Kehat et al., 2004; Xue et al., 2005); cardiomyocytes from induced pluripotent stem cells (iPSCs) also have been produced and characterized (Novak et al., 2010; Mandel et al., 2012) but not yet implanted to form a biologic pacemaker. In addition, adult mesenchymal stem cells, not driven down a cardiac lineage but genetically modified to generate a pacemaker current (If), have also proven successful (Potapova et al., 2004; Rosen et al., 2008).

A third potential application for an improved nodal-like population of stem cells would be to screen new chronotropic agents. The development of the bradycardic agent ivabradine (DiFrancesco, 2010) has demonstrated a therapeutic market for such drugs, and the existence of a spontaneously active cell line with appropriate nodal characteristics would greatly facilitate high-throughput screening of new molecules. At present, one is limited to either screening such agents against a single target channel that is a contributor to automaticity or conducting labor-intensive single-cell patch-clamp studies on SAN cells isolated from various animal preparations that may differ in unknown ways from human SAN cells. Studies have demonstrated the feasibility of recording spontaneous activity from cardiomyocytes derived from stem cells either through the use of electrodes embedded in the culture substrate (Mandel et al., 2012) or by the use of calcium-sensitive fluorescent dyes (Zahanich et al., 2011), although additional work is needed to adapt either method to a high-throughput screen and to validate these approaches on an enriched population of nodal-like cells. Finally, a variation on this is the use of patient-specific iPSCs to explore the cellular or molecular basis of those congenital cardiac disorders of complex origin (either because of a multigenic origin or because of mutation in a gene not directly related to membrane excitability) and to screen for appropriate therapeutic agents for these disorders. Such an approach has been suggested and demonstrated with ventricular-like cells derived from iPSCs of patients with diverse long QT genetic mutations (Moretti et al., 2010; Itzhaki et al., 2011; Malan et al., 2011; Yazawa et al., 2011). Recently, we reported (Nawathe et al., 2013) on a patient with long QT resulting from a mutation in a β-subunit that not only interacts with a K channel associated with long QT syndrome but also with additional K channels and with the hyperpolarization-activated cyclic nucleotide–gated (HCN) channels important to automaticity. The patient exhibited marked bradycardia, and a nodal-like cell preparation of patient-specific iPSCs might be beneficial in elucidating the mechanism(s) by which this single point mutation in a β-subunit interacts with multiple ion channels that together result in sinus bradycardia.

All these applications would benefit from a method that allows for preferential induction of nodal-like cells since current standard methods for driving stem cells down a cardiac lineage typically result in only a few percent of the cells being nodal-like; however, even if purity remains low, it may be sufficient if combined with an efficient method of selecting the nodal-like cells from within the total population. Although progress has been made recently on these fronts, the extent of ultimate success remains to be determined. Further, it is not yet known whether one cell source (adult stem cells, ESCs, iPSCs) is more suitable than another for each of the potential applications. Finally, none of the current approaches produces truly mature cardiac cells, at least with respect
to ventricular function (Yang et al., 2014). The issue is somewhat less clear in the case of nodal-like cells since in this case there is less (but nonzero) functional distinction between cells of the young and adult SAN.

In this review, we consider the current state of the art and discuss what remains to be accomplished before a preparation of human nodal-like cells derived from stem cells can be used in a range of therapeutic and basic science applications. A critical issue is how one defines and identifies cells of nodal lineage from the heterogeneous population of spontaneous cardiomyocytes originating within these stem cell preparations. In that regard, we first briefly review the critical molecular and functional characteristics of the adult SAN cell. We then review what is known about normal SAN development since this has obvious relevance to devise novel approaches aimed at preferentially driving stem cells down a nodal lineage. Finally, we review the existing literature on the preparation, selection, and characterization of nodal-like cells derived from various mouse and human stem cell sources and consider current limitations and potential future directions.

II. The Adult Sinoatrial Node

A major challenge in developing a homogeneous cell population of cells to replicate SAN functionality is that the SAN itself is heterogeneous, and that very heterogeneity—along with the nature of the connections to surrounding atrial tissue—is probably critical to the SAN’s syncytial behavior in vivo. There are differences across the nodal region in cell size, density of ionic currents, connexin (Cx) expression, and other molecular markers (Boyett et al., 2003), which may impact function. For example, superfusion of the isolated node with a β-adrenergic agonist results in a shift of the initiation site within the node, suggesting localized differences in adrenergic responsiveness (Boyett et al., 2000). Thus, at present, the best one may be able to accomplish is to settle on a collection of characteristics or cellular parameters that are most representative of the SAN as a whole and most relevant to its function and use these as screening criteria at the molecular or cellular level, recognizing that the resulting homogeneous preparation will recapitulate only a subset of the complex functionality of the syncytial SAN. Eventually, tissue engineering techniques (Camelliti et al., 2005) may allow more complete reconstruction of complex nodal tissue architecture; however, an accurate representation would require not just replicating the multicellular pattern but also any heterogeneity in cell coupling and ion channel expression, both of which vary as a function of location within the node and disease state (Kohl et al., 2005; Hao et al., 2011).

The most obvious and relevant feature of the SAN is spontaneous activity, and some clear qualitative commonalities throughout the SAN contribute to this behavior, even if there are regional quantitative differences across the tissue. One such hallmark of SAN cells that is important for their automatic function is the relative low abundance of inward rectifier K current, I\(_{\text{Kr}}\) (Cohen and Robinson, 2006). The paucity of background outward current during diastole allows a relatively small inward current to drive the SAN cell to threshold. In this regard, the second typical feature of SAN cells is the presence of the I\(_f\) which is generated by the HCN gene family, which is highly expressed in the SAN. Conceptually, this current is ideally suited for pacemaker function in that it activates on hyperpolarization (at the end of the action potential), deactivates on depolarization (thus minimally contributing to action potential duration), and directly binds the β-adrenergic second-messenger cAMP (which shifts I\(_f\) activation to less negative potentials), thereby providing autonomic responsiveness (Robinson and Siegelbaum, 2003). Whereas debate on the importance of this current continues (Lakatta and DiFrancesco, 2009), there is no question that HCN subunits (specifically HCN4) are highly expressed throughout the SAN of different species (Shi et al., 1999; Yamamoto et al., 2006; Liu et al., 2007; Brioschi et al., 2009; Chandler et al., 2009), that HCN4 mutations result in cardiac rhythm disorders (Baruscotti et al., 2010), and that an I\(_f\)-selective blocker slows (but does not stop) sinus rate (DiFrancesco, 2010). Thus, both functionally and as a molecular marker, the presence of HCN4 can be considered a minimal criterion for identifying a nodal-like cell. As detailed in section II, developmental data argue that HCN4 is a general marker of pacemaker cells rather than a specific marker of only sinoatrial cells. That is, although all nodal cells may have pacemaking functionality, not all pacemaker cells are of nodal lineage. Further, whereas I\(_f\) typically activates at less negative voltages in SAN than other cardiac regions in the adult, the same is not necessarily true in the immature heart (Robinson et al., 1997). Thus, HCN4 expression and a positively activating I\(_f\) are necessary but not sufficient selection criteria.

Other studies have explored the role of calcium homeostasis in basal and adrenergically-stimulated automaticity (Ju and Allen, 1999; Lakatta et al., 2008). Although laboratory and species differences in the magnitude of the effect were found, there is general agreement that disruption of calcium homeostasis, typically by use of ryanodine to deplete sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores, slows sinus rate, and reduces the extent to which rate increases in response to β-adrenergic agonists (Bogdanov et al., 2001; Bucchi et al., 2003). It has been argued that the basis for this contribution of calcium homeostasis to SAN automaticity is the electrogenic sodium (Na)/calcium (Ca) exchange current, which generates a depolarizing current when removing Ca\(^{2+}\) released from the SR from the cell. The primary source of the Ca\(^{2+}\) released from the SR and removed by the Na/Ca exchange current is the L-type
Ca current, \( I_{\text{Ca,L}} \), which is largely responsible for the action potential upstroke in SAN cells. In this respect, other potential identifying characteristics of the SAN cell are a high abundance of the L-type calcium channel isoform CA\(_{v}1.3\) and T-type isoforms CA\(_{v}3.1\) and CA\(_{v}3.2\) (Mangoni et al., 2003; Marianneau et al., 2005; Chandler et al., 2009; Scavone et al., 2013) and also the relative absence of fast inward Na (NA\(_{v}1.5\)) current, although the latter is not absolute; several studies have reported the presence of Na current and Na channel isoforms in SAN, depending on species and developmental stage (Baruscotti et al., 1996, 1997; Maier et al., 2003).

More recent data suggest that the \( I_{f} \) and calcium homeostasis pathways may not be independent, and that it is the adenyl cyclase (AC) signaling cascade that provides the interconnection. Unlike working myocardium, SAN expresses several AC isozymes that are activated by Ca\(^{2+}\) in the \(-100 \text{ nM} \) range, specifically, AC1 and AC8 (Mattick et al., 2007; Younes et al., 2008). In comparison, the ventricle predominantly expresses AC5/6, which are inhibited by Ca > 1 \( \mu \text{M} \). Thus, greater Ca\(^{2+}\) influx might not only increase the depolarizing Na/Ca exchange current but also activate AC1/8, thus increasing cAMP and, through it, \( I_{f} \). This observation most likely explains why disruption of calcium homeostasis also reduces the ability of \( \beta \)-adrenergic agonists to increase \( I_{f} \) (Kryukova et al., 2012). Further, the presence of Ca\(^{2+}\)-activated AC isozymes in SAN also may contribute to the fact that basal cAMP is higher in SAN than elsewhere in the heart (Vinogradova et al., 2006; Kryukova et al., 2012). This characteristic of SAN cells is itself important since it provides a means by which cholinergic agonists reduce the spontaneous rate, even in the absence of prestimulation by adrenergic agonists (DiFrancesco et al., 1989).

Much of what has been described about the molecular and ionic basis of SAN automaticity derives from animal studies, for which the rabbit is the prototypical and best characterized preparation. Considerably less data have been derived at the single cell level from human SAN, but recent reports of SAN dysfunction associated with HCN4 mutations (Baruscotti et al., 2010) and therapeutic efficacy of the \( I_{f} \) blocker ivabradine (DiFrancesco, 2010) support at a qualitative level a role for \( I_{f} \) in human SAN automaticity. Several studies from the Wilders Laboratory have provided some quantitation for human \( I_{f} \) and they report that \( I_{f} \) is present in human SAN but has a markedly lower current density and more negative activation threshold than in rabbit, compatible with the difference in heart rates between the species (Verkerk et al., 2007a,b). In an interesting analysis, they used a computer simulation to assess the current level of understanding of the functional impact of the reported HCN4 and KCNE2 (an HCN4 \( \beta \)-subunit) mutations (Verkerk and Wilders, 2014). Although the simulations indeed predicted an impact on SAN function, they also highlighted inconsistencies with the clinical data and illustrate the limited state of our knowledge concerning the cellular and molecular basis of automaticity and its autonomic regulation in human SAN.

Consideration of the specific transmembrane channels and other proteins contributing to automaticity and its autonomic regulation is obviously relevant to creating functional criteria by which to assess the “nodal” characteristics of cardiomyocytes derived from stem cells. In addition, these proteins can serve as positive or negative molecular markers either to aid in the selection or subsequent identification of cells of nodal lineage. In addition to those channel isoforms and other proteins described herein that are either highly expressed or relatively absent in the SAN, there are additional molecular markers of nodal cells such as neurofilament-M (NF-M) and the connexin isoforms Cx45 and Cx30.2 (Marianneau et al., 2005; Boyett et al., 2006; Scavone et al., 2013) as well as transcription factors that may also serve as molecular markers. These are summarized in Table 1 and described in subsequent sections.

III. Developmental Origin of the Sinoatrial Node

Ancients already knew that the heart was “endowed with life,” as Aristotle observed many centuries ago and as Claudius Galen from Pergam (some 2000 years ago) confirmed when he recognized that both ventricles pulsate even when their nerves are severed or the heart is removed from the thorax. Thus, the power of pulsation has its origin in the heart itself (Aird, 2011). It was not until 1882, however, that Gaskell proposed his theory on the myogenic origin of the heartbeat (Gaskell, 1882). Furthermore, he identified the sinus-auricle (the sinus node) as the region from which the contractions start and propagate to the ventricle-auricle (the atrioventricular (AV) node) and then to the ventricle. Finally, in 1907, 1 year after Tawara (1906) described the AV node, Keith and Flack (1907) identified the SAN in the heart of a mole and of several other animals: “The nature of this remnant (of the sinus venosus) is perhaps best exemplified in the heart of the mole. Here it is seen that at the sinoauricular junction there is a mass of remarkable tissue. It appears to the eye as a very intimate network of palely stained undifferentiated fibers with a large number of well-stained nuclei. It is totally different from the surrounding musculature.”

Although the anatomic location and the physiologic function of the sinus node were clear, at the middle of the 20th century, the embryologic origin of the pacemaker tissue was less clear. On the basis of the contraction rate of the tubular heart, it seemed that at a very early stage of development the pacemaker was located in the bulboventricular part (the prospective ventricle), and only later, when atrium and sinus venosus formed, the pacemaker shifted to this location.
In 1967, Van Mierop clearly demonstrated that in chick embryos at 8- and 9-somite stages—although hearts either beat faintly or did not visibly beat at all—it was possible to use an intracellular microelectrode to record action potentials in both the bulboventricular and sinoatrial regions, with sinoatrial beats always preceding ventricular ones (Van Mierop, 1967). A few years later, using an optical mapping technique, it was also demonstrated that spontaneous electrical activity in chick embryo can be measured in the cardiac primordia, even before their fusion to form the linear heart tube (Kamino, 1991). These data clearly demonstrated that a functional pacemaker region is already present at early stages of development. Cells constituting the primary myocardium of the early embryonic heart tube present structural and functional properties similar to those of mature nodes (sinoatrial and AV). In particular, these cells display spontaneous beating characterized by weak contractions and morphologically appear pale, with a high glycogen content and a poorly organized sarcomeric structure.

The preceding features and the lack of specific markers have delayed the identification and localization of sinoatrial precursors. One of the first markers identified to be specifically expressed in the SAN and in the other portions of the conduction system is NF-M, a protein expressed mainly in neuronal cells. Using in situ hybridization, it was demonstrated that in the adult rabbit, NF-M transcript is localized in myocytes of all the components of the cardiac conduction system, the SAN and AV nodes, and the AV bundle and bundle branches, but not in working (atrial and ventricular) cardiomyocytes. During rabbit embryonic development, NF-M mRNA is already detectable in a subpopulation of cardiac myocytes at embryonic day (E) E9.5. The expression of NF-M in the cardiac conduction system initially suggested a neuroectodermic origin of the conduction system, later proven incorrect (Gorza et al., 1988; Vitadello et al., 1996).

As shown by Van Mierop, the most important functional characteristic of a pacemaker cell is its ability to repeatedly generate the so-called prepotential or diastolic depolarization. A prominent role in the generation of the diastolic depolarization is played by the If current generated by HCN channels. Of the four known isoforms of HCN channels, HCN4 is the most expressed one in the adult sinus node of different species (Yamamoto et al., 2006; Liu et al., 2007; Brioschi et al., 2009; Chandler et al., 2009). On this basis, it has been shown that expression of HCN4 mRNA can be detected as early as E7.5 in the precardiac mesoderm (cardiac crescent) and

(Patten, 1949; DeHaan, 1965; Van Mierop, 1967). In 1967, Van Mierop clearly demonstrated that in chick embryos at 8- and 9-somite stages—although hearts either beat faintly or did not visibly beat at all—it was possible to use an intracellular microelectrode to record action potentials in both the bulboventricular and sinoatrial regions, with sinoatrial beats always preceding ventricular ones (Van Mierop, 1967). A few years later, using an optical mapping technique, it was also demonstrated that spontaneous electrical activity in chick embryo can be measured in the cardiac primordia, even before their fusion to form the linear heart tube (Kamino, 1991). These data clearly demonstrated that a functional pacemaker region is already present at early stages of development. Cells constituting the primary myocardium of the early embryonic heart tube present structural and functional properties similar to those of mature nodes (sinoatrial and AV). In particular, these cells display spontaneous beating characterized by weak contractions and morphologically appear pale, with a high glycogen content and a poorly organized sarcomeric structure.

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TABLE 1

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<th>Embryonic SAN</th>
<th>Postnatal SAN</th>
<th>Species</th>
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<sup>a</sup>Scavone et al., 2013.
<sup>b</sup>Hashem and Calycomb, 2013.
<sup>c</sup>Rust et al., 2009.
<sup>d</sup>Zhu et al., 2010.

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then in the sinus venosus (E8). During the subsequent phases of development, HCN4 expression delineates the formation of the SAN and the conduction system, even though after E10, it also can be detected in the developing nervous system (Garcia-Frigola et al., 2003; Liang et al., 2013). Thus, rather than being a specific marker of SAN precursor cells, HCN4 is most likely a specific (functional) marker of pacemaker cells. Two articles have indeed shown that HCN4 is expressed early during cardiogenesis in progenitors of the first heart field (FHF) (Liang et al., 2013; Spater et al., 2013) well before formation of the sinus venosus and SAN. Lineage-tracing experiments in mice have clearly shown that cells expressing HCN4 at gastrulation (E6.0) and at the crescent stage (E7.0) give rise, at later stages (E19.5), to compact and trabecular layers of the left ventricle and parts of both atria. HCN4 expression is then downregulated in these progenitors and starts to be upregulated in the Tbx18+ progenitors that will give rise to sinus venosus (Spater et al., 2013). Even if FHF progenitors expressing HCN4 at very premature stages (<E7.5) will not be part of the mature cardiac conduction system, they represent the spontaneously rhythmic cells that start peristaltic contraction as soon as the heart tube is formed (Christoffels et al., 2010) (Fig. 1); however, HCN4 starts to play a fundamental functional role only when its expression shifts to the prospective SAN region, as evidenced by the observation that both global and cardiac-specific HCN4 knockout mice die in utero between E9.5 and E11.5, a time matching the development of the SAN (Stieber et al., 2003). These pieces of evidence clearly show that at the cardiac level, HCN4 is a marker that specifically characterizes pacemaker cells at stages of development when the sinus node is not yet formed (<E9.5).

Besides HCN4 expression, which confers intrinsic spontaneous activity to pacemaker cells, another fundamental feature that makes pacemaker cells able to pace and to drive neighboring cells in a functional syncytium is their junctional resistance. In fact, the small spontaneously beating area (source) and the much larger excitable surrounding tissue (sink) need to have a poor electrical coupling to allow the generation and propagation of the excitatory stimulus (Joyner and van Capelle, 1986). Among the Cxs expressed in the heart, Cx45 and Cx30.2 form low-conductance gap junctions, whereas Cx40 and Cx43 form high-conductance gap junctions. Notably, gap junctions are scarce in the embryonic heart, expression of Cx40 and Cx43 is negligible (van Kempen et al., 1991; Delorme et al., 1997), and expression of Cx45 is low and widespread (Alcolea et al., 1999). This is probably due to the fact that expression of high-conductance Cxs is modulated by the same factors controlling the overall differentiation of the working

![Fig. 1.](image-url)
myocardium. It is known, for example, that expression of Cx40 and Cx43 and other genes of the working myocardium, such as Nppa, are promoted by Tbx5 and Nkx2.5, two transcription factors responsible for the specification of the working myocardium but fully complementary to the expression of Tbx3, a transcription factor specifically controlling the development of the conduction system (see later) (Hoogaars et al., 2004). Using transgenic mice, it was shown that Tbx3 deficiency causes the expansion of working myocardial gene expression (Cx40, Cx43, Nppa) into the SAN, whereas deficiency of Nkx2.5 causes ectopic expression of Hcn4 and Tbx3 and decrease of Cx40 in the atrium (Hoogaars et al., 2007; Christoffels et al., 2010). Similarly, atrial ectopic expression of Tbx3 in mice led to repression of working myocardium genes and activation of Hcn4 and Cx30.2 (Hoogaars et al., 2007; Christoffels et al., 2010). Therefore, the expression of low-conductance Cxs and the low abundance of assembled gap junctions ensure that the pacemaker cells are able to beat spontaneously and to drive the surrounding tissue during cardiac morphogenesis.

A. Elucidation of the Gene Program Leading to Sinoatrial Node Formation

With the advancement of lineage tracing techniques and the help of transgenic mice, it was possible to identify the progenitors of the sinus node cells well before its formation. Mommersteeg and colleagues (2010) have shown that the sinus venosus develops from Tbx18+/Nkx2.5+/Isl-1− progenitors that separate quite early (around E8) from the rest of the cardiac mesoderm. At E8.5, some cells among these progenitors start to express also Isl-1 (Tbx18+/Isl-1+/Nkx2.5−), and precisely this subgroup of cells will turn on the genetic pathway that leads to SAN development (Fig. 1).

With progression of embryonic development, cardiac chambers (atria and ventricles) start to develop by proliferation and the addition of new progenitor cells (Moorman and Christoffels, 2003). Two conditions must be met in the region originating the sinus node: 1) the genetic program for chamber specification needs to be inhibited; and 2) the genetic program for maintenance of pacemaker properties needs to be promoted. Two transcription factors, Tbx3 and Shox-2, have been found to be fundamental for these processes.

At E9.5, the subset of Tbx18+/Isl-1+/Nkx2.5− cells within the sinus venosus starts to express Tbx3, a transcription factor that represses chamber development by specifically inhibiting expression of the atrial genes Nppa and Cx40. Expression of Tbx3 is continuous during cardiac development in those regions that will form the mature conduction system, from the SAN to the bundle branches of the ventricular conduction system (Hoogaars et al., 2004). The importance of Tbx3 in maintaining the pacemaker features of future SAN cells is evidenced by the fact that its lack does not prevent the morphologic development of the SAN but causes ectopic expression of atrial genes in the SAN region.

Shox2 is a transcriptional repressor that, when knocked down, causes embryonic lethality from the development of a hypoplastic SAN causing an abnormally low heart rate. Interestingly, Shox2 knockdown causes lack of Tbx3 and HCN4 expression in the SAN and a parallel ectopic expression of the atrial genes nppa and Cx43. During normal cardiac development, Shox2 is expressed starting from E8.5 in the region at the junction between the common cardinal vein and primitive atrium (Espinoza-Lewis et al., 2009). Its expression becomes restricted to the inflow tract and in particular to the sinus venosus. With the progression of development, Shox2 expression is restricted to the right side of the sinus venosus in the region coinciding with the forming SAN. This expression pattern also has been demonstrated during human embryonic development (Liu et al., 2012).

Shox2, like Tbx18, has an expression pathway complementary to that of Nkx2.5, the best marker of the FHF that during development marks the whole heart, with the exception of the conduction system (Liu et al., 2012). Nkx2.5 expression turns on the genetic pathway, leading to cardiac chamber formation; it can bind to Cx40 and Nppa promoters and activate their expression (Hoogaars et al., 2004). Overexpression of Nkx2.5 in the atrium causes alteration of SAN function by inducing the expression of specific atrial genes, and for this reason, it is important to repress Nkx2.5 expression in the conduction system. Shox2 is responsible for the repression of the Nkx2.5 gene, thus protecting the SAN from activating the genetic pathway of the chamber myocardium and allowing expression of Tbx3 and HCN4, which instead are required for setting the functional properties of pacemaker cells (Espinoza-Lewis et al., 2009).

Tbx18, Isl-1, Shox2, and Tbx3 are important for specifying the region where the sinus venosus and then the SAN develop. However, whereas the sinus venosus is a symmetric structure, the sinus node develops only in the right horn of the sinus venosus. This asymmetry is ensured by the homeobox factor Pitx2c (Fig. 1). Pitx2c is not expressed in the forming sinus node; indeed, in Pitx2c-deficient mice, two SAN primordia develop, one on the right and another on the left side. This left-sided SAN primordium expresses HCN4 but lacks Cx40. At later stages (E15.5), when the four-chambered heart is formed, the left-sided SAN is morphologically similar to the right-sided SAN and expresses the same set of specific genes. Pitx2c thus specifically suppresses the SAN gene program, allowing the correct asymmetric development of the conduction system (Mommersteeg et al., 2007).

These data clearly demonstrate that pacemaker cells do not represent unspecialized primitive myocardial cells but rather derive from the activation of a specific gene pathway in progenitors that separate early during development.
cardiogenesis. This distinction is critical to efforts to produce an enriched preparation of nodal-like cells from stem cells since functionally nodal cells and immature ventricular cells are superficially similar in that both can exhibit spontaneous activity arising from a somewhat depolarized maximum diastolic potential followed by a diastolic depolarization.

B. Sinoatrial Precursor

Is it possible to identify and separate SAN precursors at early stages of development? The acquired knowledge on the genetic pathway leading to the specification of sinoatrial pacemaker cells has laid the foundations to specifically select SAN precursors. The development of specific transgenic mice has been particularly relevant to this aim. For example, transgenic mice expressing the reporter genes Lac-Z or green fluorescent protein (GFP) in the Tbx18 locus have allowed dissecting from E9.5 embryos the Tbx18+ mesenchyme destined to form the sinus venosus and to evaluate protein expression pattern in acuto or after in vitro maturation (Wiese et al., 2009; Mommersteeg et al., 2010). Interestingly, Tbx18+ mesenchyme stained negative for Nkx2.5, HCN4, and the cardiac marker myosin heavy chain (MHC; MF20) and did not show any spontaneous beating, whereas all the day-matched embryonic ventricular explants were beating. After 4 days in culture, however, a significant portion of Tbx18+ explants (around 50%) started to beat spontaneously with a rate significantly faster than that of day-matched embryonic ventricle explants. Furthermore, 4-day-old Tbx18+ explants started to express MF20 and HCN4 abundantly, but they did not express Nkx2.5, characteristic of the myocardium composing the sinus venosus.

Although these data point to Tbx18 as a promising marker for selection of SAN precursors, its use has a major drawback in that it is an intracellular marker and thus is not suitable for selecting cells without previous genetic manipulation of the organism (mouse). This drawback hampers the use of Tbx18 as a selection marker of human SAN precursors. In 2006, Hirata and coworkers showed that expression of CD166, an adhesion molecule also called activated leukocyte cell adhesion (ALCAM or DS-GRASP), marked the tubular heart and the sinus venosus at an early stage of development (E8.5), whereas its expression broadened to other organs at later stages (Hirata et al., 2006). We recently demonstrated that in mice, during cardiac development at E10.5, CD166 expression almost completely overlaps with that of HCN4; at later stages of differentiation (E12.5), HCN4 and CD166 still colocalize in the SAN region, but CD166 expression widens also to other cardiac regions and to extracardiac organs as well (Scavone et al., 2013). These data indicate that, within a well defined developmental window, CD166 can indeed identify pacemaker cells, in particular SAN precursors, even though it is not strictly a cardiac marker.

IV. Mouse Stem Cells

The possibility of regenerating the heart has become feasible with the discovery of the plasticity of stem cells. Spontaneously contracting cardiomyocytes were first detected on dimethyl sulfoxide–induced differentiation of mouse embryonic carcinoma cells (Edwards et al., 1983) and on spontaneous differentiation of mouse embryonic stem cells (mESCs) (Wobus et al., 1991). Although the ability of pluripotent stem cells to differentiate into functional cardiomyocytes is well established, both in vitro and in vivo, their clinical research and therapeutic use are hampered by their teratogenic potential and by ethical issues (Mummery et al., 2003; Behfar et al., 2007; Zhang et al., 2008).

Later, several types of stem or progenitor cells were also isolated from various postnatal tissue or organs of mesodermal origin. Mesoderm-derived adult stem cells, such as cardiac stem cells, mesenchymal stem cells (MSCs), skeletal myoblasts, hematopoietic stem cells, and endodermal progenitor cells, have been reported to possess the potential of differentiating into cardiomyocytes either when injected in vivo into the heart of animal models with a myocardial infarction (Makino et al., 1999; Orlic et al., 2001; Kajstura et al., 2005) or when cocultured in vitro in strict association with neonatal cardiomyocytes (Badorff et al., 2003; Matsuura et al., 2004; Koyanagi et al., 2005). Since the cardiogenic potential of these cells was often assessed only at the transcription level, by looking at the appearance of mRNAs for early cardiac markers, such as Nkx2.5, GATA-4, and SMA, and because mRNA appearance does not automatically translate to the induction of a real cardiogenic program resulting in the development of functional cardiomyocytes.

As of today, controversy continues about the differentiation potential of some of these adult cells (Joggerst and Hatzopoulos, 2009). Some stem cells or progenitor cells were actually shown to fuse both in vitro and in vivo with the adjacent cardiomyocytes (Matsuura et al., 2004; Avitabile et al., 2011) rather than differentiate. Even those adult stem cells that were actually able to differentiate in vitro into cardiomyocytes usually required either coculture with isolated cardiomyocytes (hematopoietic stem cells and MSCs) or treatment with agents such as methylation inhibitors and histone deacetylase inhibitors (MSCs, cardiac stem cells) (Makino et al., 1999; Badorff et al., 2003; Beltrami et al., 2003). So far, only three types of adult mouse stem cells have shown functional differentiation into spontaneously beating cardiomyocytes: mouse MSCs (Makino et al., 1999; Planat-Benard et al., 2004), mouse dedifferentiated fat cells (Jumabay et al., 2010), and mouse mesangioblasts derived from ventricular vessels and aorta (Barbuti et al., 2010).
Makino et al. (1999) showed for the first time that clonally expanded MSCs isolated from mouse bone marrow could differentiate into beating cardiomyocytes after induction with 5-azacytidine. MSCs devoid of hematopoietic cell contamination by serial passaging were treated with 3 μM 5-azacytidine. After 1 week, cells lost the fibroblast-like morphology and acquired an elongated shape; by 3 weeks in culture, cells formed a myotube-like morphology and were spontaneously beating, a feature that was maintained for the next 5 weeks. These MSC-derived cardiomyocytes expressed several cardiac proteins, such as atrial natriuretic peptide, GATA4, Nkx2.5, Mef2C, β-MHC, and, to a lesser extent, α-MHC and α-actin. The great majority of these cardiomyocytes exhibited action potentials typical of sinoatrial-like pacemaker cells, even though with further maturation in culture around 30%–40% of cells had ventricular-like action potentials (Makino et al., 1999).

In 2004, Planat-Bénard and colleagues demonstrated that a small fraction (0.02%–0.07%) of the heterogeneous adipose tissue cell population composing the vascularized stroma of adipose tissue can spontaneously differentiate into beating cardiomyocytes. These adipose tissue–derived cells, beyond expressing the typical cardiac markers Nkx2.5, GATA4, Mef2C, and atrial natriuretic peptide (ANP), and the sarcomeric proteins β-MHC, atrial and ventricular myosin light chain-2 (MLC-2v, MLC-2a), fired spontaneous action potentials showing a prominent diastolic depolarization and were also responsive to adrenergic and muscarinic stimulation (Planat-Benard et al., 2004).

Also from adipose tissue, but this time from the adipocyte fraction, came the first report of the spontaneous differentiation of pacemaker-like cells. Jumabay et al. (2010) reported that 10%–15% of dedifferentiated fat cells spontaneously differentiated into beating cardiomyocytes. A fraction of these cardiomyocytes showed some important functional and electrical features typical of pacemaker cells in that they were indeed able to fire spontaneous action potentials and to respond to adrenergic agonists with an increased rate. Moreover, these cardiomyocytes displayed synchronous sarcoplasmic Ca²⁺ release, which indirectly indicates the presence of a well organized sarcoplasmic reticulum and expression of the proteins and pumps necessary for proper calcium handling. Although a detailed molecular analysis for sinoatrial genes was not carried out, dedifferentiated fat cell–derived cardiomyocytes expressed high levels of Mef2C mRNA, a direct activator of HCN4 transcription, but only a faint signal for Nkx2.5, a known inhibitor of HCN4 and sinoatrial development in general (see section II) (Jumabay et al., 2010).

The last type of adult mouse stem cell to show a differentiation potential toward the sinoatrial-like lineage was the cardiac mesoangioblast isolated from mouse aorta and ventricles (Barbuti et al., 2010). These mesoangioblasts displayed the highest cardiogenic potential, with spontaneous differentiation rates in the range of 50%–80%, depending on the clone. Mesoangioblasts expressed several early cardiac markers (Mesp1, Nkx2.5, GATA4, Tbx5, Cx43, and ANF), together with stem cell markers (Sca-1, c-kit) already at an undifferentiated stage. Interestingly, these progenitors also expressed GATA6, Isl-1, Tbx2, and Tbx3, transcription factors specifically involved in the embryonic development of the cardiac conduction system and SAN (Davis et al., 2001; Christoffels et al., 2010). On spontaneous differentiation, triggered by lowering the serum concentration in the culture medium, mesoangioblast-derived cardiomyocytes expressed cardiac-specific proteins, ion channels, and ion currents and were indeed able to fire either triggered or spontaneous action potentials with different waveforms (Galvez et al., 2008; Barbuti et al., 2010). Around 30% of differentiating mesoangioblasts had the molecular and functional feature of sinoatrial cells. Spontaneously beating mesoangioblast-derived cardiomyocytes expressed mainly the HCN4 channels that generated an If current with kinetics resembling those of mature SAN cells. Furthermore, like SAN cells, they did not express the inward rectifying IK1 current and expressed the low-conductance junctional protein Cx45. Finally, the spontaneous rate of mesoangioblast-derived pacemaker cells was modulated by the autonomic agonists isoproterenol and acetylcholine (Barbuti et al., 2010).

A. Evidence for Possibility of Generating Sinoatrial-Like Cells from Murine Embryonic Stem Cells

While the cardiac differentiation potential of non-ESCs is still debated, the potential of mESCs to generate spontaneously beating cardiomyocytes was established almost 30 years ago and has been used by hundreds of laboratories around the world with quite similar results. The first ESC lines were generated in 1981 by Evans and Kaufman (1981); these blastocyst-derived ESC lines spontaneously differentiate in vitro to form cystic EBs, cell aggregates recapitulating the initial stages of in vivo embryonic development that show a high frequency of cardiac differentiation (Doetschman et al., 1985).

With the rapid progression of research on ESCs, protocols for efficient cardiac differentiation of ESCs have been exploited. The best described and most widely used method for generating spontaneously beating cardiomyocytes consists of culturing cells in hanging drops containing a defined number of undifferentiated mESCs (a high yield of cardiomyocytes is usually obtained plating drops containing between 300 and 500 cells) that by gravity, after 2 days, form EBs. After 4 to 5 days of culture in suspension, EBs are plated on gelatin-coated dishes and within 1 to 2 days, foci of spontaneous beating cells start to appear. Following this protocol, 80%–90% of the EBs display areas characterized by spontaneous beating (Wobus et al., 1991). This protocol represents the gold standard for cardiac differentiation of mESCs; it works independently of the mESC line used and of the
laboratory applying it, and this reproducibility is what set mESCs apart from adult stem cells.

Wobus et al. (1991) provided the first evidence that the spontaneously beating portions of the EBs generate spontaneous action potentials synchronous with cell contraction and with the slow diastolic depolarization typical of pacemaker cells. Like pacemaker cells, these ESC-derived cardiomyocytes were modulated by autonomic neurotransmitters through the physiologic β-adrenergic-AC-cAMP pathway. Subsequent work has shown that as EBs mature, cardiomyocytes become heterogeneous with respect to action potential waveform; in particular, whereas cardiomyocytes at an early differentiation stage (9–11 days) preferentially display pacemaker-like action potentials, at later stages of differentiation (16–20 days) atrial-ventricular- and sinoatrial-like action potentials can be distinguished (Maltsev et al., 1993; Hescheler et al., 1997). Abi-Gerges et al. (2000) reported for the first time that 65% of beating cardiomyocytes isolated from EBs at early differentiation stages displayed a robust If with properties similar to those of sinoatrial cells. With further development, the percentage of cells displaying If current decreased to 45%, but the current density increased. Although spontaneous activity and the presence of If are important features of pacemaker cells, since embryonic and neonatal ventricular cardiomyocytes can also spontaneously generate action potentials and express the If (Cerbai et al., 1999), these feature are not sufficient to catalog a cell as sinoatrial-like; thus, several other conditions must be considered. As described in section II, expression of HCN4 and Cx43 characterizes both sinoatrial precursors and sinoatrial cells throughout development and postnatally (Alcolea et al., 1999; Garcia-Frigola et al., 2003; Christoffels et al., 2010). In mature SAN cells, expression of HCN1 has also been reported (Ishii et al., 1999; Liu et al., 2007; Briosi et al., 2009), whereas HCN2 and Cx43 are prevalent in neonatal ventricular cells (Yasui et al., 2001).

Van Kempen et al. (2003) provided the first evidence that ESC-derived cardiomyocytes express HCN1 and HCN4 mRNA. Using a genetically modified ESC line in which GFP expression is driven by the α-MHC promoter. Yanagi et al. (2007) confirmed at the protein level that mESC-derived spontaneously beating cells diffuse express the pacemaker channels HCN1 and HCN4 and also express the T-type calcium channels Ca_{3.1} and 3.2, characteristic of SAN myocytes (Marionneau et al., 2005). Our group has shown that not only do mESCs express both HCN4 and HCN1 channels, these channels are functionally coexpressed in caveolin-3-positive cardiomyocytes (Barbuti et al., 2009), a feature previously observed in rabbit sinoatrial cardiomyocytes (Barbuti et al., 2007). Electrophysiologic analysis revealed that mESC-derived cardiomyocytes can be grouped into two distinct populations, one with a fast-activating If current and the other with slow-activating If. The existence of these two populations of pacemaker cells has been previously reported in adult mouse SAN cells (Mangoni and Nargeot, 2001), evidence that further reinforces the similarity between ESC-derived automatic cells and mature murine pacemaker cells.

It is important, however, to mention that different results have also been published concerning the expression of HCN isoforms in ESC-derived cardiomyocytes. Two studies reported that mESC-derived cardiomyocytes predominantly express the HCN2 and HCN3 isoforms both at the mRNA and protein level with faint or null expression of HCN4 and HCN1 (White and Claycomb, 2005; Qu et al., 2008). Whether these differences arise from the different ESC lines used or from the selection of particular subtypes of cardiomyocyte is presently unknown.

### B. Improvements in the Generation of Sinoatrial-Like Cells

If the fact that mESCs can spontaneously differentiate into beating EBs is well established, so is the fact that with time in culture, the number of beating EBs decreases (Wobus et al., 1991; Barbuti et al., 2009). This decrease in the number of pacemaker cardiomyocytes prompted researchers to find new approaches for enriching cultures in cardiomyocytes. Different approaches have been pursued in this direction: 1) cell-engineering approaches that use fluorescence molecules under the transcripational control of cardiac specific promoters; 2) pharmacologic approaches based on the addition of specific molecules to the culture medium to drive specific cardiac differentiation; 3) selection of sinoatrial cells based on expression of endogenous markers.

In an attempt to isolate cardiac precursors, in 2003 Hidaka et al. (2003) generated a mESC line expressing enhanced GFP (EGFP) under the control of the Nkx2.5 gene. Although Nkx2.5 is a transcription factor involved in chamber specification, the authors showed that early selection of EGFP (Nkx2.5)^+ cells allowed the isolation of a population of mixed cardiomyocytes that, based on electrophysiologic features, represent ventricular-atrial and sinoatrial-like cardiomyocytes. This can be explained by the fact that homologous recombination used to generate the EGFP (Nkx2.5) clone inactivated one allele; indeed, these ESC cells express only half of the Nkx2.5 expressed by “wild-type” ESC cells. As expected by the role of Nkx2.5 in cardiac development, after 28 days of culture, only a small fraction of EGFP-selected cells still showed spontaneous activity. Despite the fact that this approach was not devised to isolate pacemaker cardiomyocytes, it demonstrated the possibility of actually enriching the cardiomyocyte population as evidenced by the fact that 98% of flow cytometry-selected cells stained positive for myosin heavy chain and troponymosin (Hidaka et al., 2003). In this same work, the authors also...
demonstrated that a pharmacologic approach can alter the proportion of a specific subpopulation of cardiomyocytes; for example, exogenous administration of retinoic acid (10^-7 M) in the culture medium during EB differentiation preferentially induced the atrial gene program (Hidaka et al., 2003).

A second approach to isolate a specific subtype of cardiomyocyte from ESCs was pursued by Gassanov and colleagues (2004), who generated an mESC line in which the ANP promoter drove EGFP expression. Analysis of EGFP-positive cells revealed the presence of cells with a triangle, spindle, and round morphology in the proportion of around 20%, 60%, and 20%, respectively. Triangle-shaped cells were either quiescent or fired at a relatively slow rate (around 1 Hz) and displayed action potentials with morphology and characteristics (resting potential, overshoot, and duration) typical of atrial-like cardiomyocytes. All spindle-shaped cells fired instead spontaneous action potentials at significantly higher rate (close to 3 Hz) and showed the marked slow diastolic depolarization typical of sinus node cells. In agreement, GATA6, a b-subunit functionally interacting with HERG or KvLQT1 channels to give rise to native If, and If slow, currents, respectively, is expressed in the sinoatrial-conduction system regions both during development and in the adult heart (Kupershmidt et al., 1999). GATA6 has been instead found in the developing AV conduction system (Davis et al., 2001; Adamo et al., 2004). Using these cell lines, the authors found that beating EBs showed cells coexpressing GATA6 and minK near the contracting area and that their separation prevented the contraction of the EB, suggesting that they were the pacemaker region. From an electrophysiologic point of view, GATA6/minK cells displayed heterogeneous properties because even though they expressed If current, both sinoatrial- and atrial-like action potentials were recorded. This heterogeneity becomes even clearer when the molecular profile of a pure population of GATA6-expressing cells, selected for their resistance to neomycin (resulting from a neomycin-resistance cassette under the GATA6 enhancer), was analyzed; cGATA6 cells expressed moderate to high levels of ventricular and atrial Nkx2.5, Cx43, HCN2, and MLC-2a mRNAs; very low levels of the atrial ANF and Cx40 mRNAs; moderate levels of the conduction system mRNA Tbx3, Cx45, and CaV1.3; very low levels of the sinoatrial mRNA HCN4 and HCN1; curiously, very low levels of minK mRNA were detected (White and Claycomb, 2005).

As we have pointed out, HCN4 is a fundamental and specific functional marker of the developing and mature SAN and conduction system (Garcia-Frigola et al., 2003; Morikawa et al., 2010). Morikawa et al. (2010) and, more recently, our group (Scavone et al., 2013), generated ESC lines in which EGFP expression was driven by the HCN4 promoter. Differentiation of these cell lines gave rise to EBs with a strong EGFP signal in the spontaneously beating regions; these same EGFP+ regions expressed HCN4 and other proteins, such as caveolin-3, HCN1, and CaV3.2, characteristic of mature SAN cells. Although promising, the selection strategy based on the activity of the HCN4 promoter failed to yield a pure or enriched population of sinoatrial-like cells; in fact, most of the EGFP+ cells, selected by flow cytometry, were quiescent. A possible explanation could be that, since EGFP was clearly detectable only starting from day 7 of differentiation and peaked around day 13, HCN4 started to be expressed also by other cell types (such as neuronal precursors), as demonstrated during normal embryonic development (Garcia-Frigola et al., 2003); indeed, this seems to be the case because the EGFP-selected...
population expressed low levels of the neuronal marker nestin (Morikawa et al., 2010). Moreover, since HCN4 is also expressed in FHF progenitors, sorting of HCN4+ cells obtained either from mouse embryos or human ESC results in a high percentage of ventricular- and atrial-like cells and only a minor percentage of nodal-like cells (Spater et al., 2013). These data clearly show the difficulty of finding a single selection marker that by itself recognizes a specific cell type, mainly because many genes are turned on and off at different phases of cardiac development (Christoffels et al., 2010).

More recently, Hashem and Calycomb (2013) engineered mESC with a vector containing the neomycin-resistance gene under the control of a promoter region of the Shox2 gene. This approach allowed them to obtain Shox2-expressing cells by selection with a culture medium supplemented with a high concentration of neomycin. The surviving cells, despite expressing cardiac markers Tbx5, GATA4, α-cardiac actin, α-skeletal actin, Mlc-2a, Mlc-2v, and desmin, expressed several genes of the sinoatrial/conduction system program, including Tbx2 and Tbx3, GATA6, Cx45, Cx30.2, CaV1.3, CaV3.1, HCN4, and HCN2. At the same time, the ventricular and atrial genes Nkx2.5 and ANF were only modestly expressed. However, no functional data have yet been shown that could ultimately identify these cells as sinoatrial-like (Table 1).

As good as these selection methods are, they require the manipulation of the cell genome that in the best case scenario requires inactivation of one of the allele pairs, causing a haplo insufficiency and, in the worst case, the random insertion of exogenous DNA that makes the method unsafe for future clinical use. Apart from the approaches just described, nongenomic pharmacologic approaches have also been developed to improve cardiac differentiation from ESCs. Although not initially thought to push specifically toward the pacemaker lineage, some of these approaches ended up showing a specific enrichment in sinoatrial-like cells.

As already described, the first demonstration came from the work of Hidaka et al. (2003) using retinoic acid to push cardiac differentiation toward the atrial lineage. A similar attempt, but in the pacemaker direction, was done by Gassanov et al. (2004) using an ANP-EGFP ESC line. They found that incubating ESCs from the beginning of the differentiation protocol to day 14 with the cytokine endothelin-1 (ET-1) dose dependently increased the proportion of the spindle-shaped EGFP+ cells corresponding to sinoatrial-like cells. With 100 nM ET-1, differentiation of sinoatrial-like cells increased significantly, becoming 30% of the total EGFP+ cardiac population; at the same time, triangle-shaped atrial-like cells decreased from 60% to 40%. ET-1 treatment also increased the expression level of Cx45 and Cx40 but not that of the ventricular
isoform Cx43 (Gassanov et al., 2004). The ET-1 effect was very specific because coincubating cells with the selective ET-A and ET-B receptor antagonists BQ123 (cyclo(ό-Trp-D-Asp-Pro-d-Val-Leu)) and BQ788 (N-(cis-2,6-dimethyl-1-piperidinyl)carbonyl)-4-methyl-l-leucyl-1-(methoxy carbonyl)-d-tryptophyl-d-norleucine) completely prevented the ET-1-mediated increase in sinoatrial-like cells. This work provides the first proof of the possibility of pharmacologically pushing mESC differentiation toward the nodal phenotype, even though the enrichment was still poor in terms of possible applications.

Later, Kleger et al. (2010), while studying the role of Ca\(^{2+}\)-activated potassium channels during cardiac development, found that mESC treatment with EBIO (1-ethyl-2-benzimidazolone), a drug that keeps previously activated Ca\(^{2+}\)-activated potassium (SK) channels in the open state, increased 4- to 5-fold their cardiac commitment. Based on immunofluorescence and flow cytometry analyses, they found that cells expressing a-actinin increased from 15% in control conditions to 65% with 1 mM EBIO; similarly, troponin-positive cells increased from 11% to 60%. Also, almost 60% of these EBIO-treated cardiomyocytes displayed pacemaker-like action potentials and expressed the \(I_f\) compared with 7% of nontreated cardiomyocytes. Molecular analysis showed that most of the EBIO-treated cardiomyocytes showed a robust expression of HCN4 on the plasma membrane and of Cx30.2 between cells. The enrichment in sinoatrial-like cells is further supported by the fact that EBIO treatment caused a significant upregulation of Tbx2, Tbx3, Shox2, and Isl-1, transcription factors deeply involved in the specification of the cardiac conduction system. These effects were mediated by the SK4 isoform, the most abundantly expressed isoform in the atrium and conduction system, and in differentiating mESC. The specific involvement of SK4 was demonstrated using clotrimazole, a specific SK4 inhibitor, and using a specific small hairpin RNA to knock down expression of the channel. In fact both interventions were effective in preventing EBIO-mediated cardiac differentiation and in preventing upregulation of HCN4 channels (Kleger et al., 2010).

Finally, Wiese et al. (2011) tested on differentiating ESCs the effect of suramin, a compound known to interact with specific growth factors and cytokine receptors and, at high concentrations, to induce differentiation of heart structures in Xenopus embryos (Grunz, 1992). Transient treatment with 0.5 mM suramin from day 5 to day 7 during EB differentiation increased the yield of sinoatrial-like cells that, as assessed by electrophysiologic analysis of action potential waveform, constituted between 50% and 70% of the total cardiomyocytes. In agreement with functional data, suramin treatment doubled the expression levels of HCN4 and Tbx3 two well known markers of sinus node cells. At the same time, suramin prevented the differentiation of mESC toward the neuronal and skeletal muscle phenotypes (Wiese et al., 2011).

This and the other pharmacologic approaches, even if they do not provide a sufficiently pure population of sinus node–like cells, have the great advantage of not requiring manipulation of the ESC genome and so, if combined with a proper selection or isolation method, may become attractive from a clinical point of view.

We have stated at the beginning of section IV.B that ESCs have the advantage of promptly differentiating into cardiomyocytes, but they are also particularly interesting for regenerative applications because of their high self-renewal capacity that ensures an unlimited growth potential in vitro. Their proliferation and differentiation potential, however, represent also the greatest disadvantage of ESC because of the teratogenic risk that accompanies pluripotency. For this reason, even though the proposed pharmacologic approaches represent quite effective methods to enrich ESC-derived cardiomyocytes in sinoatrial-like cells, they do not prevent the contamination of undifferentiated, potentially teratogenic cells. On the other hand, modifications of the cell genome so as to express reporter genes specifically in the lineage of interest allow specific selection of the cell of interest but are of little interest with respect to future clinical applications. The ideal approach would consist of specific selection of the cell lineage of interest using native antigens to be used for cell sorting. Unfortunately, as of today, proteins that recognize specifically sinoatrial cells or even cardiomyocytes in general are not known.

So far, the only two surface markers found to be expressed both in the heart and in ESC-derived cardiomyocytes are CD166 (or ALCAM or DS-GRASP) (Hirata et al., 2006; Rust et al., 2009; Lin et al., 2012; Scavone et al., 2013) and CD172a (or signal-regulatory protein \(\alpha\) [SIRPA]) (Dubois et al., 2011). The first evidence of the expression of CD166 in the developing heart of mice came from work that compared the transcriptome of cardiac progenitor cells with that of noncardiac cells (Masino et al., 2004). Using transgenic mice expressing enhanced yellow fluorescent protein under the transcriptional control of an enhancer element of the transcript factor Nkx2.5, they isolated by flow cytometry an almost pure population of cardiac progenitors at three developmental stages: E7.75, E8.5, and E9.5. CD166 was one of those genes specifically enriched in cardiac progenitors and clearly marked the linear heart of E8.5 embryos (Masino et al., 2004). In 2007, Murakami et al. (2007) demonstrated that CD166 expression can be used to select from E8.5 yokl sark cardiovascular precursors that in culture form clusters of cells showing spontaneous beating. Based on these data and on our data showing that CD166 was specifically coexpressed with HCN4 at early developmental stages (E10.5) but not at later stages, our group recently produced a protocol that allows the isolation of a pure population of SAN precursors from differentiating ESCs (Scavone et al., 2013). CD166+ ESCs, differentiated according to the hanging drop method, were sorted between day 6 and day 8 of differentiation, the critical
time window in which CD166 was highly specific for sinoatrial precursors. Cells sorted by a fluorescence-activated cell sorter were reaggregated by gravity in low-adhesion dishes for 24 hours and then plated. The resulting CD166-selected cells formed a spontaneously beating syncytium that can be cultured for up to 1 month. Already from the beginning, CD166 + cells expressed high levels of the transcription factors that govern SAN development, such as Tbx18, Shox2, Tbx3, isl-1, and several structural and functional proteins that drive SAN cell function such as the pacemaker channels HCN4 and HCN1, the calcium channels CaV1.3 and CaV3.2, the connexin isoforms Cx30.2 and Cx45, and the skeletal isoform of troponin I slow (ssTnI). At the same time, CD166 + cells expressed low levels of the atrial and ventricular genes Nkx2.5, HCN2, Cx43 Myh6, and Mlc2v (Table 1). Besides these molecular features, when CD166-selected cells were compared with native mouse sinoatrial cells, their functional properties were quite similar, in particular with respect to the contribution of the If and L- and T-type calcium currents to their electrical activity. Interestingly, CD166-selected cells after 3 weeks of culture coexpress HCN4 and caveolin3 and assume the spindle-shaped morphology typical of native sinoatrial cells, a feature never observed before in vitro in stem cell–derived cardiomyocytes (Fig. 3).

The only other nongenomic approaches to select an enriched population of cardiomyocytes from pluripotent stem cells used tetramethylrhodamine methyl ester perchlorate (TMRM), fluorescent molecules specifically marking mitochondria (Hattori et al., 2010) and SIRPA (or CD172) (Dubois et al., 2011). Both methods yielded a highly pure population of cardiomyocytes but without a clear specificity for a particular subtype. TMRM in fact was effective in selecting cardiomyocytes from all the species analyzed (mouse, marmoset, rat, and human), but the fact that it recognized Nkx2.5+/α-actinin + cardiomyocytes at various developmental stages suggests a lack of specificity for sinoatrial-like pacemaker cells (Hattori et al., 2010). Similarly, since SIRPA is expressed both in human atrial and ventricular cardiomyocytes from the fetal stages to adulthood, it may not be a selection marker for sinoatrial-like cardiomyocytes. Moreover, SIRPA was not detected in the mouse heart, indicating that its expression is not conserved during evolution (Dubois et al., 2011). Unfortunately, because functional data were not provided in either the TMRM or SIRPA reports, it remains to be established if one of these selection methods may be effective at a particular differentiation time point in enriching the population of sinoatrial-like cells, so CD166-based selection appears at present to be the best method available.

V. Human Stem Cells

The human equivalent of mESCs, human embryonic stem cells (hESCs), were first obtained in 1998 by
Thomson and colleagues from human embryos produced by in vitro fertilization (Thomson et al., 1998). In the following few years, translation and adaptation of the differentiation protocol developed with mESCs succeeded in obtaining human cardiomyocytes from hESCs. At present, however, a universally reproducible protocol, like the hanging drop method developed for mESCs, is still lacking. In 2001, Kehat at al. (2001) demonstrated for the first time that hESCs can develop spontaneously contracting regions that can be maintained in culture for at least 5 weeks. At early stages, contracting regions were composed mainly of round-shaped mononucleated cells of around 10–30 μm in diameter, similar to early pacemaker cells found in mESCs (Gassanov et al., 2004; Kolossov et al., 2005). Even though these hESC-derived cardiomyocytes expressed several cardiac markers (GATA4, Nkx2.5, cTnI, cTnT, MLC-2a and -2v, α-MHC, and atrial natriuretic factor), presented spontaneous and repetitive calcium transients, and were also able to respond appropriately to adrenergic and muscarinic stimulation, no information on specific subtypes of action potentials were provided (Kehat et al., 2001).

In 2003, two studies separately showed that, similar to mESC, hESCs differentiate into cardiomyocytes with action potentials typical of ventricular-, atrial-, and also pacemaker-like cells (He et al., 2003; Mummery et al., 2003) (Fig. 4). In 2004, Satin et al. measured for the first time ionic currents and ion channel expression from hESC. They found that spontaneously beating hESC-derived cardiomyocytes express robust INa and If currents but not the inward rectifying current IK1. Curiously, they stated that the automatice of these cells was mainly dependent on INa because whereas application of 3μM TTX slowed the beating rate and 10 μM TTX stopped it completely, neither nifedipine nor 2 mM Cs2+ (which block ICaL and If, respectively) had any effect on the action potential rate. mRNA analysis revealed that the hESC-derived cardiomyocytes abundantly expressed the typical ventricular isoforms Nav 1.5, Cav 1.2, and HCN2 of the sodium, calcium, and f channels, respectively, but they did not express the typical sinoatrial isoforms Cav 1.3 and HCN4, suggesting that the analysis focused on immature ventricular cardiomyocytes rather than nodal-like pacemaker cells (Satin et al., 2004). Later, Sartiani et al. (2007) carried out a study comparing spontaneously beating hESC-derived cardiomyocytes. They found that although If is expressed in most beating cells, inhibition of this conductance by the specific blockers zatebradine and ZD 7288 (4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride) significantly decreased the rate in only a subset (58%). Furthermore, they showed that pacemaker activity was blocked by inhibition of ICaL by nifedipine but was not sensitive to block of sodium channels with 10 μM TTX. This evidence and the fact that they found a relatively high expression of HCN2, HCN4, Cav1.3 NCX-1, and Tbx3, which are abundant in human SAN and perinodal areas (Chandler et al., 2009), suggest that these cells are early pacemaker or sinoatrial-like cells rather than immature atrial or ventricular cardiomyocytes.

Weisbrod et al. (2013) also found that all the hESC-derived spontaneously beating cells at early stages of differentiation functionally expressed the Ca-activated potassium channel SK4, and specific inhibition of this channel by either clotrimazole or TRAM-34 completely stopped spontaneous beating. Even though at present no data on the expression of SK4 in native SAN cells are available, the fact that in mESC activation of this channel with EBIO significantly increased differentiation of sinoatrial-like cells (Kleger et al., 2010) and the fact that at later stages of hESC differentiation only 30% of the beating cells are responsive to TRAM-34 (Weisbrod et al., 2013) suggest that expression of SK4 channels may provide a novel way to distinguish functionally sinoatrial-like cells from immature working cardiomyocytes.

Regardless of the relatively low abundance of sinoatrial-like cells derived from hESC, it is important to mention that when the beating portions of hESC-derived EB were coupled in vitro to neonatal ventricular cardiomyocytes or injected in vivo in the hearts of swine (Kehat et al., 2004) and guinea pig (Xue et al., 2005), a regular spontaneous rhythm was induced. Even though the injected cellular
substrates were not selected and thus quite heterogeneous regarding cell composition, and despite the fact that the ionic bases underlying the pacemaking activity were not addressed, it is likely that sinoatrial-like cells contributed to the generation of spontaneous rhythmic activity, as evidenced by the fact that perfusion of the f-channel blocker ZD 7288 reduced the spontaneous rate and perfusion of isoproterenol was effective in increasing rate (Kehat et al., 2004; Xue et al., 2005).

A. Human Embryonic Stem Cell-Derived Cardiomyocyte Selection and Sinoatrial-Like Cell Enrichment

Not only do hESCs differ from mESCs in the lack of a fully reproducible differentiation protocol for obtaining beating cardiomyocytes, but also the difficulty of generating hESC lines expressing reporter genes under specific cardiac promoters is holding back the specific selection of sinoatrial-like cells. In 2007, Huber et al. (2007) showed that the generation of stable hESC lines expressing EGFP under the transcriptional control of the α-MHC (MYH6) promoter. After at least 11 days of differentiation, selection with puromycin generated a population consisting 91% of spontaneously beating cardiomyocytes, which were kept in culture for up to 6 weeks and were responsive to isoproterenol stimulation. It is interesting to note that whereas the selection of α-MHC-EGFP mESC-derived cells results in the specific enrichment of atrial- and sinoatrial-like cells (Kolossov et al., 2005), hESC-derived puromycin (α-MHC)-selection results in a heterogeneous population of cardiomyocytes displaying ventricular-, atrial-, and sinoatrial-like action potentials (Anderson et al., 2007).

The aim of the preceding studies was to enrich a general cardiomyocyte population. Regarding enrichment methods for nodal-like cells, an advantage at least in terms of potential therapeutic applications (e.g., biologic pacemakers) is that the required number of cells for implantation will be far smaller than for cardiac repair. Zhu et al. (2010) focused their attention on enriching and selecting such a population of sinoatrial-like cardiomyocytes. The selection procedure was based on exposure of differentiating cardiomyocytes to a lentiviral vector containing the EGFP reporter under the control of the cGATA-6 promoter, which allows the visualization of nodal-like cells (White and Claycomb, 2005; Barbuti et al., 2010). Their data clearly demonstrate that cGATA6-GFP+ cardiomyocytes presented several properties typical of nodal-like cells. From a molecular point of view, cGATA6-GFP+ cells expressed high levels of HCN4 together with other cardiac markers (cardiac troponin T, sarcomeric actin, β-MHC), whereas from a functional point of view, 95% of EGFP+ cells displayed nodal-like action potentials and expressed a robust I1 current. Furthermore, like nodal cardiomyocytes, cGATA6-GFP+ cells displayed a low proliferative potential. The enrichment procedure was instead based on the inhibition of the pathway activated by the growth factor neuregulin 1β and its receptors ErbB (β/ErbB). Treatment of hESC cells at day 5 of differentiation with antibodies against neuregulin 1β or with the ErbB antagonist AG1478 (N-(3-chlorophenyl)-6,7-dimethoxyquinazolin-4-amine) caused a premature appearance of spontaneously beating cells, increased by almost 3-fold the number of cardiomyocytes with nodal-like action potentials and expressed a robust I1 current. Furthermore, like nodal cardiomyocytes, cGATA6-GFP+ cells displayed a low proliferative potential. The enrichment procedure was instead based on the inhibition of the pathway activated by the growth factor neuregulin 1β and its receptors ErbB (β/ErbB). Treatment of hESC cells at day 5 of differentiation with antibodies against neuregulin 1β or with the ErbB antagonist AG1478 (N-(3-chlorophenyl)-6,7-dimethoxyquinazolin-4-amine) caused a premature appearance of spontaneously beating cells, increased by almost 3-fold the number of cardiomyocytes with nodal-like action potentials (from around 20% to 50%–60%) and induced a significant upregulation of genes characteristic of sinoatrial cells such as Tbx3, HCN4, and CACNA1G (the Cav3.1 T-type calcium channel) while concurrently decreasing the working myocardium genes CACNA1C (Cav1.2 L-type calcium channel) and NaV1.5 sodium channel (Zhu et al., 2010). To date, this approach remains the most effective in generating sinoatrial-like cells from hESC. Unfortunately, as is the case with many of the approaches applied to mESC (see section IV), the selection

![Fig. 4. Action potentials in hESC-derived beating cardiomyocytes and isolated human fetal ventricular and atrial cells. Reproduced with permission from Mummery et al., 2003.](image)
strategy suffers from the use of a lentiviral infection procedure that might not be suitable for future therapeutic applications (despite the small number of cells implanted), although it would be entirely appropriate for in vitro drug screening applications. On the other hand, more recent approaches avoid random integration, for example, the use of zinc-finger technology to target the AAVS1 locus on chromosome 19 (Hockemeyer et al., 2009).

Cell-selection approaches using extracellular antigens have also been exploited using both hESC and human iPSC, even though these strategies were not devised for selecting pacemaker-like cardiomyocytes. The selection procedure using CD166 that works so well in selecting sinoatrial-like cells from differentiating mESC (Scavone et al., 2013) was actually first used on hESCs for enriching the population of cardiomyocytes (Rust et al., 2009) and later on human iPSCs (Lin et al., 2012). Rust and colleagues, for example, have sorted CD166+ cells from human ESCs after 12 days of differentiation, a stage at which expression of CD166 and other cardiac markers (Nkx2.5, Gata4, Isl-1, MeF2C, Tbx5, and α-MHC) was highest. Interestingly, CD166-selected cells expressed high levels of the HCN1 and HCN4 pacemaker genes and of the CACNA1C L-type calcium gene (see Table 1). Even though a detailed analysis of specific sinoatrial genes and electrophysiologic data were not provided, the evidence that reaggregated human CD166-selected cells keep beating spontaneously for at least 3 weeks suggests that CD166 may represent a good marker of sinoatrial-like cells (Rust et al., 2009). CD166 has also been used to select a population of cells enriched in cardiomyocytes from differentiating human iPSCs previously selected for the cardiovascular marker KDR; also in this case, however, despite the evidence that more than 90% of CD166+ cells expressed the cardiac troponin T protein (but also smooth muscle actin), no functional data on action potential waveform or ionic currents were provided (Lin et al., 2012).

Two other extracellular proteins possibly suitable as selection markers for sinoatrial-like cells are HNK-1 (also known as Leu-7 or CD57) and VCAM1 (or CD106). HNK-1 has been shown to transiently mark the sinus venous and other precursors of the cardiac conduction system during human and rat development (Blom et al., 1999; Wenink et al., 2000); interestingly, in humans, HNK-1-positive precursors give rise also to the myocardiurn surrounding the pulmonary veins, a locus often involved in the generation of abnormal ectopic atrial automaticity (Blom et al., 1999). Besides this evidence, it is not presently known whether it can be used to select pacemaker-like cells from human pluripotent stem cells. The second possible marker is VCAM1. Using hiPSC-derived cardiomyocytes, Uosaki et al. (2011) found that VCAM1 was the only protein among other 242 markers to be coexpressed with the cardiac isoform of troponin T (TNNT2) and, indeed, VCAM1-positive cells, selected at differentiation day11, expressed several cardiac markers and displayed both ventricular- and pacemaker-like action potentials.

It is worth noting that all the markers mentioned (CD166, HNK-1, and VCAM1) are not typical cardiac proteins; instead, they are nonspecific adhesion molecules transiently expressed during development. This evidence suggests that timing of selection may be as important as the marker(s) used for selection.

In the future, it will be interesting to understand if a combination of the pharmacologic and selection approaches described herein could succeed in isolating a pure population of sinoatrial-like cells from pluripotent cells of human origin.

**B. Human Induced Pluripotent Stem Cells and Automaticity**

Human induced pluripotent stem cells have been produced for a number of years (Yu et al., 2007), and further progress has been made in recent years in terms of the methods and diversity of tissue sources, as well as in driving these cells down a cardiac lineage. A detailed discussion of protocols and potential pitfalls in the production and use of human iPSCs is beyond the scope of this article but has been the subject of recent reviews and editorials (Yoshida and Yamanaka, 2010, 2011; Mummery, 2011; Xu et al., 2011; Sinnecker et al., 2012). With respect to the specific issue of electrophysiologic characteristics of these cells, there also have been several recent reviews (Poon et al., 2011; Hoekstra et al., 2012; Zeevi-Levin et al., 2012). In the following discussion, we focus specifically on reports concerning aspects of automaticity and the presence of nodal-like cells in these preparations since that is most relevant to the current topic.

Zhang et al. (2009) used OCT4, SOX2, NANOG, and LIN28 transgenes to reprogram human iPSCs obtained from fetal and newborn tissue samples and compared the results both molecularly and electrophysiologically with the H1 and H9 hESCs lines. They reported recording action potentials with ventricular, atrial, and nodal-like characteristics from both preparations, and the relative percentage of each cell type was similar in hESCs and hiPSCs. In both, most of the cells were ventricular-like (~60% of ESCs and ~72% of iPSCs) and nodal-like cells represented 10%–20% of the population (13% and 20% in the 2 iPSC lines studied and 11 and 20% in the two ESC lines). The spontaneous rate also was similar in both preparations, as well as chronotropic responsiveness to isoproterenol. However, although they measured various cardiac and sarcomeric markers, they did not test for the expression of sinoatrial-specific markers.

In 2011, Ma et al. (2011) conducted a similar study on iPSCs derived from a human fibroblast cell line, although without a direct comparison with hESC lines. They also reported a mix of ventricular, atrial, and
nodal-like cells in similar proportion to the earlier study (54% ventricular, 22% nodal). As with the prior study, the definition of nodal-like cells was based entirely on action potential parameters. Although they did also measure individual currents, including the I_f, they appear to have restricted such studies to the ventricular-like cells in their population.

Lee et al. (2011) also compared hESC and two hiPSC cell lines (IMR90 and KS1) but they focused on the Ca^{2+}-handling characteristics of the cells, a key aspect of sinoatrial automaticity. They found that the Ca^{2+}-sinoatrial automaticity. They found that the Ca^{2+}-handling characteristics of hiPSCs were immature relative to those of hESCs. In particular, calcium transients were smaller and had slower upstroke velocities. Most importantly, ryanodine was less effective in reducing the calcium transients in the hiPSCs. Surprisingly, Lee and colleagues reported a higher percentage of nodal-like cells (again based on action potential characteristics) than earlier studies, both in hiPSCs and hESCs (45% and 43%, respectively).

A recent study reported on the derivation and characterization of cardiomyocytes from iPSCs from keratinocytes obtained from adult human hair samples (Mandel et al., 2012). Besides the advantage of a readily accessible tissue source from adults, this study compared automaticity and beat rate variability in hiPSCs and hESCs. They found the beating rates of both preparations were similar and were stable during the 15-day study period. They also reported that both preparations exhibited similar intrinsic beat rate variability, as previously reported in human populations.

Overall, the current knowledge concerning the nodal-like nature of cardiomyocytes derived from hiPSCs, and in particular hiPSCs from adult tissue sources, is limited. The results suggest that cells with nodal-like action potentials are certainly present in these populations, but it remains to be determined whether these are cells of true nodal lineage or simply immature “ventricular-like” cells with pacemaker activity. The one report of immature calcium handling in hiPSCs (Lee et al., 2011) also raises a cautionary note that merits further study.

It should be noted that a number of other studies have used patient-specific iPSCs to study cardiomyocytes from diseased individuals, including LEOPARD syndrome (multiple lentigines syndrome), familial dilated cardiomyopathy, and long QT syndrome (Carvajal-Vergara et al., 2010; Sun et al., 2012; Terrenoire et al., 2013). This is a promising future direction in terms of furthering understanding these diseases and perhaps developing more targeted therapeutic approaches but does not directly relate to the issue of producing an enriched population of nodal-like cells. Similarly, once nodal-like cells (of either iPSC or ES origin) can be routinely prepared, opportunities also arise for studying acquired sinus dysfunction. However, full realization of this potential may require production of more complex in vitro preparations involving mixed cell types (i.e., fibroblasts and atrial-like cells along with nodal-like cells) since in situ nodal function depends on such heterogeneous structures and pathology involves both structural and electrical remodeling (Kohl et al., 2005; Oren and Clancy, 2010; Hao et al., 2011).

VI. Conclusions

Much of the research on the derivation of cardiomyocytes from various stem cell sources has been directed at ventricular-like cells, which in fact represents the majority of the cells in the typical heterogeneous cell population. Certainly, such cells have tremendous therapeutic potential in terms of improving cardiac mechanical function in the diseased heart. Research on pacemaking characteristics has been more recent and to a large extent directed toward the potential use of such cell preparations as source material for creating a biologic alternative to electronic pacemakers. Unfortunately, since our knowledge on the genetic pathway leading to SAN formation has been partly clarified only in the last decade, there has not always been a distinction made between pacemaker-like and nodal-like cells, and so it remains uncertain the extent to which these preparations, which have reported “nodal-like” action potentials in anywhere from a few percent to as high as 45% of the cells, truly contain cells of nodal lineage. This distinction is not trivial with respect to the biologic pacemaker application since such a pacemaker based on immature but automatic cells of ventricular lineage might subsequently become excitable but non-spontaneous mature ventricular cells.

Furthermore, because most of the research and protocols for stem cell differentiation toward nodal lineage have been developed in mouse cells, only a few studies have attempted to select or enrich specifically for human cells of nodal lineage, based on intracellular or surface markers. These results are promising, but much more progress needs to be made both on translating mouse research to human and in determining the optimal markers to use, as well as developing methods to enrich the preparations for these markers. If these efforts prove successful, there would be a diverse range of applications for a human purified cell line of nodal lineage that recapitulates the key characteristics of SAN pacemaking.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Barbuti, Robinson.

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