Embryonic Stem Cell–Derived CD166+ Precursors Develop Into Fully Functional Sinoatrial-Like Cells

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Rationale: A cell-based biological pacemaker is based on the differentiation of stem cells and the selection of a population displaying the molecular and functional properties of native sinoatrial node (SAN) cardiomyocytes. So far, such selection has been hampered by the lack of proper markers. CD166 is specifically but transiently expressed in the mouse heart tube and sinus venosus, the prospective SAN.

Objective: We have explored the possibility of using CD166 expression for isolating SAN progenitors from differentiating embryonic stem cells.

Methods and Results: We found that in embryonic day 10.5 mouse hearts, CD166 and HCN4, markers of the pacemaker tissue, are coexpressed. Sorting embryonic stem cells for CD166 expression at differentiation day 8 selects a population of pacemaker precursors. CD166+ cells express high levels of genes involved in SAN development (Tbx18, Tbx3, Isl-1, Shox2) and function (Cx30.2, HCN4, HCN1, CaV1.3) and low levels of ventricular genes (Cx43, Kv4.2, HCN2, Nkx2.5). In culture, CD166+ cells form an autorhythmic syncytium composed of cells morphologically similar to and with the electrophysiological properties of murine SAN myocytes. Isoproterenol increases (+57%) and acetylcholine decreases (~23%) the beating rate of CD166-selected cells, which express the β-adrenergic and muscarinic receptors. In cocultures, CD166-selected cells are able to pace neonatal ventricular myocytes at a rate faster than their own. Furthermore, CD166+ cells have lost pluripotency genes and do not form teratomas in vivo.

Conclusions: We demonstrated for the first time the isolation of a nonteratogenic population of cardiac progenitors able to mature and form a fully functional SAN-like tissue. (Circ Res. 2013;113:389-398.)

Key Words: cardiac progenitor cells ■ embryonic stem cells ■ HCN channels ■ pacemaker ■ sinoatrial node

The sinoatrial node (SAN) is the natural pacemaker of the heart. Pacemaker cells are specialized myocytes, lacking a stable resting potential, which at the end of an action potential generate a diastolic or pacemaker depolarization that drives the membrane potential slowly up to the threshold for firing the next action potential.

In the past 2 decades, the increasing implantation rate of electronic pacemakers has been primarily attributable to isolated sinus node dysfunction.1 With increasing population aging, dysfunctions of the conduction tissue, which may trigger threatening arrhythmias, are expected to become more and more common.

With the advent of gene-based and cell-based therapeutic approaches, researchers have focused their efforts on the development of a biological pacemaker that is a cellular substrate able to connect to and induce ectopic spontaneous activity in the host tissue.2,3 Ideally, a biological pacemaker should be composed of cells identical to SAN cardiomyocytes. Cells with pacemaking properties may be generated from pluripotent stem cells (embryonic stem cells [ESCs] and induced pluripotent stem cells).4,5 Unfortunately, the high self-renewal capacity and plasticity of pluripotent stem cells, which make them interesting for regenerative purposes, represent their greatest disadvantages because these features imply a high teratogenic potential. There is evidence that in vitro cell commitment and differentiation of ESCs would eliminate the risk of teratoma formation.6,7 A limiting step in developing therapeutic applications using pluripotent stem cells consists in the isolation of a homogeneous population of cells with the desired phenotype. For

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example, we and others have shown that mouse ESCs differentiate into cardiomyocytes with the molecular and functional features of mature pacemaker cells; these cells are scarce and interspersed with other cell types.\(^4\) So far, most of the approaches used to isolate cardiomyocytes from pluripotent stem cells involved their genomic modification with reporter genes,\(^9,10\) which makes these cells barely suitable for future clinical applications. Alternative selection methods are hampered by the lack of specific extracellular cardiac markers exploitable for cell sorting. However, there are data showing that CD166 is specifically but transiently expressed in the developing mouse heart, including the sinus venosus,\(^11,12\) the region from which the SAN develops, and that CD166 can be used to enrich human ESCs in immature cardiomyocytes.\(^13\) This evidence has led us to hypothesize that CD166 expression could represent a suitable marker to select precursors of pacemaker cardiomyocyte during a specific differentiation stage.

In this work, we have developed a protocol to isolate a population of CD166\(^+\) pacemaker precursors from differentiating murine ESCs, and we have shown that these cells develop into a spontaneously beating layer of cells, expressing many of the molecular and functional markers characterizing the mature SAN cells.

### Methods

Detailed Methods are available in the Online Data Supplement. The procedures used in this work conform to National and European directives for the care and use of laboratory animals (D.L. 116/1992; 86/609/CEE). Animal protocols were reviewed and approved by the local Institutional Review Board and by the Italian Ministry of Health.

#### ESC Culture and Differentiation

Mouse ESCs (D3 line, ATCC, and CGR8 line) were grown and differentiated as embryoid bodies (EBs) as previously described.\(^4\) Mouse ESCs (D3 line, ATCC, and CGR8 line) were grown and differentiated as embryoid bodies (EBs) as previously described.\(^4\) Differentiating mouse ESCs were prepared as previously described.\(^4\) For flow cytometry analysis and sorting, 6-, 8-, 10-, and 15-day-old differentiated murine ESCs, and we have shown that these cells develop into a spontaneously beating layer of cells, expressing many of the molecular and functional markers characterizing the mature SAN cells.

#### Flow Cytometry Sorting

For flow cytometry analysis and sorting, 6-, 8-, 10-, and 15-day-old EBs were collected, enzymatically and mechanically dissociated, and incubated with the fluorophore-conjugated antibodies following manufacturer instructions. Analyses were performed either soon after the sorting procedure or after 24 hours of cell reaggregation.

#### Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Gene expression was quantified by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR; Line-GeneK, Bioro) using SYBER Premix Ex Taq II (Takara), 50 ng cDNA, and 500 mmol/L primers (for primers see Online Table I). Data are expressed as \(2^{ΔΔCt}×100\). Because of the large range of values among the populations, statistical analysis was performed with the logarithm \(2^{ΔΔCt}×100\).

#### Electrophysiology

Spontaneous action potentials were recorded by the patch-clamp technique in current clamp mode using the whole-cell configuration. Temperature was kept at 36±1°C. Isoproterenol and acetylcholine were added to the extracellular solution at the proper concentration from stock solutions. For voltage-clamp recordings, only single cells were used. Solutions and voltage protocols were previously described.\(^4\)

### Immunofluorescence and Video-Confocal Analysis

Samples were fixed in 4% paraformaldehyde, permeabilized, and incubated over night at 4°C with primary and fluorophore-conjugated secondary antibodies as previously described.\(^4\) Confocal images were acquired using a video-confocal microscopy ViCo (Nikon).

#### Statistics

One-way ANOVA followed by Fisher least significant difference mean comparison or Student \(t\) test for independent populations was used as appropriate. Significance level was set at \(P=0.05\).

### Results

#### CD166 Is Coexpressed With HCN4 During Heart Development

So far, CD166 is the only marker also detected in the sinus venous, the prospective SAN.\(^11,12\) Here, we have evaluated the expression pattern of CD166 in the developing mouse conduction system/SAN, identified as the cardiac regions expressing the pacemaker channel HCN, as previously reported.\(^14,15\) As shown in Figure 1, in developing hearts of embryonic day 10.5 mouse embryos, CD166 and HCN4 signals are almost completely overlapped (Figure 1A–1C). At embryonic day 12.5, HCN4 and CD166 still colocalize in the region corresponding to the developing SAN (Figure 1D–1F); however, as previously reported,\(^11\) CD166 expression broadens and becomes more evident in ventricles (Figure 1E) and in several extracardiac organs and tissues (Online Figure I). These data support the use of CD166 as a good candidate to isolate pacemaker cell precursors at early developmental stages.

#### CD166 Identifies Cardiac-Committed Cells in Differentiating Mouse ESCs

We performed flow cytometry analysis and sorting of cells dissociated from EBs and labeled with an anti-CD166 antibody at various differentiation time points; qRT-PCR was then used to quantify the expression of sarcomeric \(α\)-actinin to establish when CD166 specifically identifies cardiac-committed cells. Figure 2A shows representative dot plots of the population of viable cells (P1) obtained from the dissociation of EBs and labeled with an anti-CD166 antibody at various differentiation time points; qRT-PCR was then used to quantify the expression of sarcomeric \(α\)-actinin to establish when CD166 specifically identifies cardiac-committed cells. Figure 2A shows representative dot plots of the population of viable cells (P1) obtained from the dissociation of EBs and labeled with an anti-CD166 antibody at various differentiation time points; qRT-PCR was then used to quantify the expression of sarcomeric \(α\)-actinin to establish when CD166 specifically identifies cardiac-committed cells. Figure 2A shows representative dot plots of the population of viable cells (P1) obtained from the dissociation of EBs and labeled with an anti-CD166 antibody at various differentiation time points; qRT-PCR was then used to quantify the expression of sarcomeric \(α\)-actinin to establish when CD166 specifically identifies cardiac-committed cells.
Figure 1. CD166+ expression in the developing cardiac conduction system. A and D, Hematoxylin and eosin staining of embryonic day 10.5 (A) and embryonic day 12.5 hearts. Confocal images of adjacent slices of embryonic day 10.5 (B, C) and embryonic day 12.5 hearts (E, F) showing the expression of CD166 and HCN4 proteins. Nuclei stained by 4',6-diamidino-2-phenylindole (DAPI). Calibration bar, 200 μm. Avc indicates atrioventricular canal; la, left atrium; lv, left ventricle; ra, right atrium; rscv, right superior caval vein; rsh, right sinus horn; and rv, right ventricle.

We also evaluated the expression of genes whose expression is specifically associated with the endodermal, ectodermal, and noncardiac mesodermal lineages in CD166+ cells. The qRT-PCR analysis revealed that in CD166+ cells, the expression levels of the ectodermal marker synaptophysin, the endodermal marker transthyretin, and the mesodermal skeletal muscle–specific marker myoD were low (Online Figure III), further indicating that CD166 recognizes a specific subpopulation of cardiac precursors.

CD166+ Cells Express Typical SAN Genes

After sorting and 24 hours of reaggregation, most of the CD166+-derived aggregates started to beat spontaneously (Online Video I) and continued to beat vigorously in culture (Online Video II) for ≤4 weeks. Spontaneous contraction was never seen in aggregates derived from CD166- cells. We quantified the fraction of cells expressing α-actinin and HCN4 at various days after sorting (days 2, 3, and 4; Online Figure IV). α-Actinin was expressed in 77% to 87% of the CD166+ cells and in 15% to 17% of the CD166- cells, whereas HCN4 was expressed in 82% to 84% of the CD166+ cells and in 1% to 16% of CD166- cells (Online Table II shows actual values).

We then used qRT-PCR to compare the mRNA levels of several genes expressed either in the embryonic and adult SAN or in ventricles with the levels found in early (just after sorting) or in late (3–4 weeks in culture) CD166+ cells (Figure 4). We first analyzed the expression of the transcription factors Tbx18, Tbx3, Isl-1, and Shox2, which are important in SAN formation.21–23 The expression of these genes was high in early CD166+ cells and, although it decreased in late cultures, remained at levels comparable with those found in the SAN and at levels significantly higher than those found in the ventricle.

We next quantified the gene expression of several proteins and ion channels essential for SAN function.24 In CD166+ cells and SAN, most of these genes (ssTnI, HCN4, HCN1, caveolin 1.3, Cx30.2) are expressed at significantly higher levels than in the ventricle. Expression of the T-type calcium channel (CaV3.2), which is high in early CD166+ cells, decreased significantly at later stages; nevertheless, this decrease was accompanied by a slight increase of the CaV3.1 isof orm, the other T-type calcium isof orm expressed in the SAN;24,25 a similar isof orm switch between CaV3.2 and CaV3.1 has been previously documented during both mouse development and mouse ESC differentiation.26,27 Connexin 45, an isof orm found in all cardiac regions of the embryonic heart and downregulated after birth,24,28 was expressed at similar levels in all groups except in early CD166+ cells, where its expression was significantly higher. Finally, in CD166+ cells, we quantified the expression of typical ventricular genes, such as Nkx2.5, Kv4.2, HCN2, and Cx43, and again we found levels similar to those of the SAN rather than those of the ventricle (Figure 4). Taken together, these data indicate that during their in vitro maturation, CD166-selected cells display changes in the gene expression profile, which largely recapitulate that of the native SAN cells.

In a subset of experiments, we also have evaluated the expression of HCN4, HCN1, ssTnI, and Shox2 in CD166+ cells; as expected, these markers were significantly less expressed than in CD166- cells (Online Figure V).
HCN and Calcium Channels Are Functional in CD166-Selected Cardiomyocytes

Because HCN channels are critical for pacemaker activity in both embryonic and adult SAN,29,30 and because their ectopic expression induces repetitive spontaneous activity in the ventricle,31 we evaluated the expression of various HCN subunits at the protein level after 1 or 3 weeks in culture (Figure 5). In agreement with qRT-PCR data, we found that after 1 week in culture, CD166+ cells expressed both HCN1 (Figure 5B) and HCN4 (Figure 5E) together with the cardiac markers α-actinin (α-act; Figure 5A and 5D), whereas HCN2, the main ventricular isoform,32 could not be detected in α-act–positive cells (Figure 5G and 5H). Figure 5I shows a staining of rat neonatal ventricular myocytes as a positive control of HCN2 staining. Interestingly, after 3 weeks in culture, CD166-selected cells acquired a morphology similar to that of adult SAN myocytes (Figure 5J, 5K, 5M, and 5N) and, like adult SAN cells, showed colocalization of HCN4 and CaV3 staining (Figure 5L). Furthermore, like SAN, CD166-selected cells expressed negligible levels of the atrial-specific α-myosin heavy chain (Myh6) and of the ventricular-specific myosin light chain 2v (mlc2v), suggesting no progression of differentiation toward a chamber phenotype (Online Figure VI).

The distribution of HCN4 staining in 25-day-old CD166-selected cell syncytia was similar to that observed in murine SAN slices (Online Figure VII).

Because CD166+ cells express the HCN isoforms (HCN4 and 1), typically found in the SAN, we also analyzed the If current. CD166-selected cells display the If current both after 1 week (Figure 6A, left) and 3 weeks in culture with a voltage dependence comparable with that of native mouse SAN cells (Figure 6A, right); V 1/2 were −77.4±2.4 mV and −73.4±1.2 mV in early (n=7, filled circles) and late (n=3, open circles) CD166-selected cells, and −73.6±1.4 in SAN myocytes (n=9, open squares).30

We also evaluated the contribution of the L- and T-type calcium currents (ICaL, ICaT) to spontaneous activity of CD166-selected cells by superfusing the ICaT blocker Ni2+ (50 µmol/L) and the ICaL blocker nifedipine (0.1 µmol/L) during spontaneous action potentials recordings (Figure 6B); on average, the rate of Ni2+ decreased by 17.7±2.6% (n=5) and the rate of nifedipine decreased by 16.9±6.4% (n=3); these values were not significantly different from those obtained from mouse SAN cells (Ni2+: 21.6±7.5, n=5; nifedipine: 16.5±2.2, n=6).

As mentioned previously, CD166-selected cardiomyocytes maintained their spontaneous activity for >3 weeks in culture;
during this period, their beating rate increased (Figure 7A), and the increase was comparable with that of normal mouse embryonic development, suggesting a certain degree of maturation.

Finally, we evaluated whether CD166-selected cardiomyocytes were competent to respond to autonomic agonist stimulation, a feature of SAN myocytes important for modulation of cardiac chronotropism. In Figure 7B, confocal images of CD166-selected cells double-stained with anti β1-adrenergic, β2-adrenergic, or anti-M2 muscarinic (M2 acetylcholine) receptors and CaV3 or α-actinin (as indicated) are shown. In Figure 7C, spontaneous action potentials before (control) and during superfusion of the β-adrenergic agonist isoproterenol (1 μmol/L) or the muscarinic agonist acetylcholine (0.1 μmol/L) are shown. Upon isoproterenol and acetylcholine stimulation, the beating rate increased by 56.9±8.0% (n=5) and decreased by 22.9±5.4% (n=3), respectively.

**HCN4 Promoter Is Active and Delineates CD166+ Cells**

We also generated a clone of ESCs, stably expressing the enhanced green fluorescent protein (EGFP) under the transcriptional control of the HCN4 promoter (pHCN4-EGFP; Online Data Supplement). The pHCN4-EGFP-derived EBs displayed EGFP-positive contracting portions coexpressing CaV3 and HCN4 (Online Figure VIIIA). As expected, when CD166+ cells were selected from pHCN4-EGFP EBs, the whole beating layer (Online Video III) showed the EGFP signal (Online Figure VIIIB).

**CD166-Selected Cells Drive the Rate of Cocultured Neonatal Ventricular Myocytes**

We have then evaluated whether CD166-selected cells can function as a pacemaker; to this aim, we have used a widely used coculture system. Rat neonatal ventricular myocytes were plated on top of spontaneously beating CD166+ cells or on top of quiescent CD166−, or they were plated alone. After a few days, when a syncytium was formed, the spontaneous rate was calculated from recordings of action potentials. Cardiomyocytes in coculture with CD166+ cells had a mean rate of 1.7±0.19 Hz (n=5; data not shown), which is significantly higher than the rate obtained from cardiomyocytes in coculture with CD166− (0.8±0.17 Hz; n=6) and from cardiomyocytes alone (0.8±0.09 Hz; n=7). These data indicate that CD166+ cells are able to electrically couple and drive an excitable substrate, thus behaving as a biological pacemaker.

**CD166+ Cells Have a Low Proliferative Potential In Vitro and Are Not Teratogenic In Vivo**

One of the major drawbacks of pluripotent stem cells resides in their high proliferative and differentiation potential, leading to teratoma formation. We have quantified CD166+ cell proliferation by bromodeoxyuridine staining; CD166+ cells showed a low proliferative potential at 24 hours (7.2±2.7%), a value that further decreased at 48 hours (3.8±1.8%); as expected, the bromodeoxyuridine incorporation rate of undifferentiated ESCs was high (63.5±4.9%). Furthermore CD166+ cells, unlike mES cells, failed to induce teratomas when injected in vivo in CD1 nude mice (Online Data Supplement and Online Figure IX).

**Discussion**

The possibility to generate de novo a population of stem cell–derived pacemaker cardiomyocytes similar, if not identical, to mature SAN cells would be highly desirable for the development of either a cell-based therapeutic approach aimed at reestablishing the proper cardiac rhythm (biological pacemaker) or as an in vitro cell/tissue model for testing cardioactive drugs. Pluripotent stem cells are particularly attractive for this aim because they can generate spontaneously beating cells with the molecular and functional features typical of SAN/pacemaker myocytes. Because pacemaker cells originating from differentiating ESCs are interspersed among other cell types, their specific selection and isolation remain a major challenge.

So far, 2 markers have been found to be expressed both in the heart and in ESC-derived cardiomyocytes: CD166 (or ALCAM) and CD172a (or SIRPA). Because pacemaker cells originating from differentiating ESCs are interspersed among other cell types, their specific selection and isolation remain a major challenge.

The fact that SIRPA is expressed in both the fetal and the adult human heart (in both atria and ventricles), and the fact that in human ESCs SIRPA can be used to select a subpopulation enriched in cardiac troponin T–positive precursors, rules against its use as a selection marker for pacemaker precursors. Furthermore, SIRPA could not be detected in mouse heart, suggesting that it does not represent an evolutionary conserved protein for cardiac development.
We have selected cells expressing CD166. Although this protein is not an established cardiac marker, it is transiently but specifically expressed in the developing heart tube and sinus venosus, the prospective SAN.11,12 Here, we have shown that CD166-expressing cells also express HCN4 (Figure 1), a protein that specifically delineates the SAN region during embryogenesis14,15,41 and in adulthood.42–44 HCN4 is also fundamental for sinus node function because its absence is incompatible with life29,30 and mutations in its sequence can cause inherited rhythm disturbances.46

Here, we show that freshly selected CD166+ cells, but not CD166− cells, express high levels of HCN4 mRNA; at 1 day after sorting, most of the cells express the HCN4 proteins (Online Figure IV). After 4 weeks in culture, CD166-selected cells maintain a high HCN4 expression and also assume the spindle-shaped morphology typical of SAN cells forming.

Figure 4. Comparison of gene expression in early and late CD166+ cells, sinoatrial node (SAN), and ventricle. Quantitative reverse-transcriptase polymerase chain reaction analysis of transcription factors (Tbx18, Tbx3, Isl-1, and Shox2), structural proteins, and ion channel (ssTnl, HCN4, HCN1, CaV1.3, CaV3.2, CaV3.1, Cx 30.2, and Cx 45) involved in SAN development and function and of ventricular genes (Nkx2.5, Kv 4.2, HCN2, and Cx43). *P<0.05 vs SAN; #P<0.05 vs ventricle.
cell agglomerates like those of native SAN (Figure 5J–5N and Online Figure VI).

CD166+ cells also express high levels of Tbx18, Shox2, Tbx3, and Isl-1, which are known to characterize the pool of mesodermal progenitors that separate early during cardiogenesis to create the sinus venosus and then the SAN. Isl-1 also identifies a population of HCN4-expressing myocytes in both the adult murine and human SAN, but it is not expressed in the working myocardium. After 3 weeks in culture, the expression level of these genes is still comparable with that found in the SAN, and is higher than levels found in the ventricle. Thus, our data show that CD166 represents an optimal marker for specifically selecting SAN precursors.

CD166-based selection has been previously used to isolate cardiovascular precursors from yolk sac. At embryonic day 8.5, yolk sac–derived CD166+ cells create both endothelial and cardiac cells; however, if CD166+ cells are also selected for flk-1, only the CD166+/flk-1− population formed cardiomyocytes. In agreement with this evidence, our data show a negligible coexpression of CD166 and flk-1.

However, other data show that ESC-derived flk-1+ cardiac precursors also express c-kit and sca-1, and a subpopulation of ESC-derived flk-1+ cardiac precursors selected by sca-1 formed α–myosin heavy chain–positive contracting aggregates. CD166+ cardiac precursors do not express significant levels of either flk-1 or Sca-1 and express very low levels of c-kit (Figure 3). Whether this means that our CD166+ cells represent a different population or a different stage of the same population of precursors, it remains to be elucidated.

Previously, Rust et al have shown that CD166 selection of differentiating human ESCs results in a population enriched in cells defined as embryonic cardiomyocytes on the basis of ion channels and structural protein expression. We have instead found that mouse ESC–derived CD166+ cells represent a population specifically committed to become SAN myocytes, as indicated by the expression of specific genes (Figure 4) and proteins (Figure 5) and by the electrophysiological properties typical of native adult SAN cells (Figure 6).

Differences between our data and those of Rust et al could arise from the different species used and, more likely, as a consequence of the different isolation protocols. For example, they isolated cells when CD166 expression peaked; we instead selected cells when the difference in α-sarcomeric actinin expression between CD166+ and CD166− cells was maximal. Other important differences concern the cell proliferation potential of CD166-selected cells. They found a proliferation rate of ≈20% to 30%, whereas our CD166-sorted cells have a much lower proliferation rate (<4% at 48 hours from sorting). Moreover, their cells maintain high CD166 expression for ≥2 weeks, whereas in our cells CD166 expression decreased significantly (Online Figure VIII A), as expected during cardiomyocytes maturation.
Previous attempts to generate pacemaker/SAN myocytes from ESCs have used either a cell-engineering or a pharmacological approach, but neither one resulted in a homogeneous population. Morikawa et al. generated mouse ESCs expressing the EGFP under the transcriptional control of the HCN4 gene promoter. Contracting areas of these engineered EBs expressed EGFP, HCN4, and other cardiac proteins; however, when EGFP+ cells were sorted, the majority did not show spontaneous action potentials but instead expressed the neuronal marker nestin, which is in line with the fact that neuronal cells also express HCN4 channels. Pharmacological treatment of differentiating ESCs either with 1-ethyl-2-benzimidazolinone or with suramin increased the fraction of sinus node–like cell population by 6-fold and 3-fold, respectively; however, these treatments did not prevent contamination by other cell lineages.

In addition to the gene expression pattern, our CD166-selected cardiomyocytes also display the functional properties peculiar to the SAN cardiomyocytes. The main function of SAN myocytes is to spontaneously generate action potentials and to modulate cardiac rhythm to account for changes in oxygen demand. CD166-selected cardiomyocytes form a synchronously beating syncytium (Online Videos II and III), which fires repetitive and regular action potentials. It is interesting to note that CD166-selected cardiomyocytes, like embryonic hearts, increase their beating frequency with time, reaching a rate similar to that previously reported for dissociated adult mouse SAN myocytes. The similarity between CD166-derived syncytia and mature SAN is further validated by the presence of functional β-adrenergic and cholinergic pathways (Figure 7) that physiologically modulate the response to autonomic stimulation and by the electrophysiological features analyzed. In particular, our data clearly show that the kinetic properties of If and the effect on rate exerted by the blockade of calcium channels are the same in freshly isolated sinoatrial myocytes and CD166-selected cells.

Finally, it is important to note that a major concern regarding the use of pluripotent cells relates to their ability to easily generate teratomas in vivo, unless they are committed or differentiated before injection. Based on the low proliferation potential, the lack of expression of pluripotency-related genes, and the failure to induce teratomas when injected in CD1 nude mice, CD166+ cells represent a safe substrate.

Study Limitation
Despite our CD166-selected cells forming a spontaneously beating syncytium and being able to electrically connect to and pace a monolayer of ventricular myocytes in vitro, their abilities to engraft within the heart and to drive it at physiological rates remain to be addressed. Similarly, although heart development is quite conserved in mammals, the possibility of translating the present protocol to human-induced pluripotent stem cells, even though CD166 expression has been reported in the human heart and in human ESC-derived cardiomyocytes and induced pluripotent stem cells–derived cardiomyocytes, remains to be elucidated.

Conclusions
In conclusion, we have demonstrated that it is possible to specifically recognize and select mesodermal precursors expressing several transcription factors characterizing those cells that will originate the sinus node in the embryo. Thus, we can speculate that once these precursor are isolated from other
lineages of the EBs, this condition favors a high level of expression of transcription factors, such as Tbx18, Shox2, and Tbx3, preventing the switch-on of gene programs leading to the working myocardium. Thus, the CD166-selected cells maintain the molecular features necessary to develop into fully functional SAN cardiomyocytes. This finding, when applied to human pluripotent stem cells, could pave the path for the development of a cell-based biological pacemaker useful for clinical and in vitro pharmacological applications.

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Disclosures
None.

References


During early heart development, CD166 is coexpressed with the HCN4 channel and connexin45. Selection of embryonic stem cell–derived cardiomyocytes is hampered by the lack of specific extracellular markers. In the mouse, CD166 is transiently expressed in the developing heart.

**What New Information Does This Article Contribute?**

- During early heart development, CD166 is coexpressed with the HCN4 subunit of the pacemaker channels, the best known marker of the sinusoidal node (SAN).
- Cultured CD166- cells, selected from differentiating mouse embryonic stem cells, express high levels of many genes involved in SAN development and function but express low levels of genes of the working myocardium, thus representing bona fide precursors of pacemaker cells.
- Cultured CD166-selected cells acquire the morphological and functional properties of mature SAN myocytes and are able to electrically drive an excitable cellular substrate, thus behaving like a biological pacemaker.

Dysfunctions of the conduction tissue could trigger life-threatening arrhythmias that are often treated by implantation of electronic pacemakers. Electronic devices, however, present several limitations that may be overcome by developing a stem cell–based biological pacemaker. Here, we report the development of a protocol based on the selection of CD166-expressing cells that allows the recognition and isolation of SAN precursors from embryonic stem cells. CD166- cells express several genes involved in SAN development and present the typical molecular and electrophysiological properties of mature SAN cardiomyocytes. CD166-selected cells also are able to pace in vitro a substrate of newborn ventricular myocytes, thus behaving as a pacemaker. The translation of these findings to pluripotent stem cells of human origin could aid the development of a biological pacemaker useful for in vitro pharmacological testing and for future clinical applications.
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Detailed Methods

Mouse embryo tissue sections staining
Mouse embryos at developmental stage E10.5 and E12.5 were isolated from CD1 females’ mice, collected in cold PBS and fixed in 4% paraformaldehyde (PFA) for 1 or 2 hours respectively. The embryos were washed twice in PBS, dehydrated by washing in PBS containing increasing concentration of sucrose (10, 20 and 30%), included in O.C.T™ compound and frozen in liquid nitrogen-cooled isopentane. Serial 8-µm-thick sections were cut with a Leica cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Cryosections were incubated in PBS with 1%BSA and 0.1%TritonX-100 for 1 hour at room temperature (RT) and then blocked with 10% donkey serum in PBS for 30 minutes at RT. The specimens were incubated with the primary antibody (anti-CD166, Santa Cruz Biotechnology, Inc. 1:50 or anti-HCN4 Santa Cruz 1:50) overnight at 4°C followed by secondary staining, for 1 hour at RT, with Alexa Fluor® (488) donkey anti-goat secondary antibody, diluted 1:250 in PBS. Slides were then washed three times in PBS, incubated with Hoescht (1mg/ml for 5min at RT), washed, mounted using Fluorescence Mounting Medium (Dako) and analyzed at fluorescence microscopes (Leica).

Cell culture and differentiation.
Mouse ES cells (D3 line, ATCC, and CGR8 line) were grown in suspension in ES-medium containing ES-DMEM (ATCC), 15% Knock-Out Serum Replacement (Gibco), 0.1 mmol/L Non Essential Amino Acids (Gibco), 0.1 mmol/L β-mercaptoethanol (Sigma), 2 mmol/L L-glutamine (Gibco), 10^5 U/ml Leukemia Inhibitory Factor (LIF, Chemicon), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Euroclone).

ES cells engineering.
For the generation of the cell clone constitutively expressing the nuclear LacZ (nLacZ) reporter gene, ES cells were generated as previously described. Briefly, ES cells colonies were dissociated to single cells and then incubated overnight (O/N) with 1 ml of third generation nLacZ (CMV promoter) lentiviral suspension at MOI 1 with polybrene (8 µg/ml, Sigma Aldrich). After one day cells were centrifuged, washed once in PBS (Euroclone) and then cultured in growing medium. Lentiviral particles were produced and titered as previously described. The pHCN4-EGFP clone was obtained as follows: a 841 bp sequence located 2.30 Kb upstream of the 5’ ATG of the HCN4 gene, and corresponding to its promoter (pHCN4), was cloned from mouse genomic DNA and inserted into the expression vector pEGFP-N1 (Clontech) after removal of the constitutive CMV promoter. The resulting pHCN4-EGFP plasmid carries the enhanced green fluorescent protein (EGFP) under the transcriptional control of the pHCN4. 2x10^6 mouse ES D3 cells were nucleofected (Mouse ES nucleofector kit VPH-1001, A24 program, Amaxa) with 10 µg of pHCN4-EGFP plasmid and plated in 3 100 mm dishes in ES-medium. To induce plasmid integration, cells were kept under stringent selection (600 µg/ml of geneticin) for at least two weeks, changing the medium every two days. After this period, the geneticin concentration was lowered to 300 µg/ml.

Flow cytometry and cell sorting
For flow cytometry analysis and sorting EBs were collected and washed twice in PBS, incubated with Tryple Express (Gibco) for 10 min at 37°C under gentle agitation, and then mechanically dissociated. The cell suspension was centrifuged at 310 x g for 15 min and resuspended in PBS plus 1 mmol/L CaCl₂, 10% FBS and 5 mmol/L EDTA. Cells were incubated with the fluorophore-conjugated antibodies for 30 min on ice, in the dark and under gentle agitation, following manufacturer’s instruction. The antibodies used were: APC rat anti-mouse Flk-1 (BD Biosciences), PE-Cy7 rat anti-mouse c-kit (BD Biosciences), FITC rat anti-mouse Sca-1 (BD Biosciences), APC rat anti-mouse CD90.2 (BD Biosciences) V450 rat anti-mouse CD44 (BD...
Biosciences), PE-Cy7 Rat anti-mouse CD34 (Santa Cruz), PE Rat anti-mouse CD166 (eBioscience). After incubation, cells were washed twice in PBS and resuspended in PBS plus 1 mmol/L CaCl₂, 5 mmol/L EDTA, filtered through a 70 µm sterile mesh and analyzed by a FACSAria™ flow cytometer and sorter (BD Biosciences). For each antibody used, the appropriate isotype control and unstained cells were used to set the FACS conditions. For subsequent analyses cells were used just after the sorting procedure (day8) or were re-aggregated by gravity for 24h in low-adhesion culture dishes in differentiating medium and subsequently plated at either high density to allow the formation of a compact layer or low density for electrophysiological analysis, in silicon inserts (Giemme). After about 3 hours, the inserts were removed.

**Neonatal cardiomyocytes (co-)cultures.**

Neonatal rat cardiomyocytes were isolated as previously described. For co-culture experiments, either 2.5x10⁵ re-aggregated CD166+ or CD166- were plated in square silicon supports (0.03 cm², Ibidi). After 4 hours the support was removed and the culture medium was changed the next day. After 3-4 days, 8x10⁵ rat neonatal ventricular myocytes were plated, in a delimited area using a cloning cylinder, both on top of CD166+ or CD166- cells and on a distal empty area in the same dish. The inserts were removed after about 3 hours. Co-cultures were kept in differentiating medium for at least 3-4 days before electrophysiological analysis.

**Teratoma Assay**

All animal procedures were conformed to Italian law and approved by the San Raffaele Animal Care and Use Committee (IACUC 355). CD-1 nude mice were housed in Charles River Laboratories and/or at San Raffaele Scientific Institute animal house in specific pathogen-free (SPF) conditions. ES cells or FACS-sorted ES-selected CD166+ cells were resuspended in 100 µl of diluted Matrigel™ (1:10, Cultrex) and injected with a 20G syringe (BD) in the left dorsal flank of CD-1 nude mice. After the development of the teratoma, mice were euthanized and the teratoma processed for further analysis (see the section below).

**Hematoxilin & eosin and x-gal stainings**

Teratomas were fixed O/N in 4% paraformaldehyde (Sigma) at 4°C under gentle agitation. Small fragments were dehydrated in growing concentration of sucrose (7%, 15% and 30% in PBS), to remove water, and then included in OCT (Tissue-Tek) with isopentane (VWR). 8µm-thick tissue sections were cut at the cryostate and stained with hematoxylin/eosin (H&E) (Sigma) and X-gal (Invitrogen) according to standard protocols.

**RNA extraction and quantitative (q)PCR**

Total RNA was extracted with Trizol (Invitrogen). 1 µg of RNA from each sample was treated with DNase I (Fermentas) to eliminate residual generic DNA and reverse transcribed using the Superscript KIT (Invitrogen) with random hexamers following the manufacturer’s protocol. Gene expression was quantified by real-time qPCR (Line-GeneK, Bioer); experiments were carried out using SYBER® Premix Ex Taq™ II (Takara) using 50 ng of cDNA and 500 nmol/L of each primer. All primer pairs had efficiency ≥ 95%. qPCR conditions were: first step at 50°C for 2 min, second step at 95°C for 20 sec followed by 45 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The melting curve analysis (60°C to 96°C) was performed at the end to verify the presence of a single amplicon. Each sample was performed in triplicate and the mean cycle threshold (Ct) of the gene of interest was normalized to the mean Ct of the housekeeping (hk) gene. For α-actinin quantification, experiments were performed using both the hypoxanthine guanine phosphoribosyl transferase (HPRT) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, data not shown) as hk genes; since α-actinin expression pattern was similar with both hk genes, expression of all the other genes was quantified using only HPRT.

**Electrophysiology**

For electrophysiology, cells were placed onto the stage of an inverted microscope and were superfused with Tyrode solution containing (mmol/L): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5.5 D-glucose, 5 Heps-NaOH; pH 7.4. Patch-clamp pipettes had resistances of 3-6 MΩ when filled with the intracellular-like solution containing (mmol/L): 130 K-aspartate, 10 NaCl, 5 EGTA-KOH, 2 CaCl₂, 2 MgCl₂, 2 ATP (Na-salt), 5 creatine phosphate, 0.1 GTP (Na-salt), 10 Heps-KOH; pH 7.2. Spontaneous action potentials were recorded by the patch-clamp technique in current clamp mode using the whole-cell configuration.
Temperature was kept at 36±1°C. Isoproterenol (Iso) and acetylcholine (ACh) (Sigma) were added to the Tyrode solutions at the proper concentration from concentrated stock solutions. For voltage-clamp recordings only single cells were used. 1 mmol/L BaCl2 and 2 mmol/L MnCl2 were added to the Tyrode solution to improve dissection of Iκ. To record Iκ, hyperpolarizing test steps to the range -45/-125 mV were applied from a holding potential of -35 mV, followed by a fully activating steps at -125 mV. Each test step was long enough to reach steady-state current activation. Activation curves were obtained from normalized tail currents measured at -125 mV and fitted to the Boltzmann distribution: 
\[ y = \frac{1}{1+\exp((V - V_{1/2})/s)} \]
where \( V \) is voltage, \( y \) fractional activation, \( V_{1/2} \) the half-activation voltage, and \( s \) the inverse-slope factor.

**Immunofluorescence and video-confocal analysis**

Samples were fixed in PFA (4%) on ice for 30 minutes, rinsed for 20 min with PBS containing 0.1 mol/L glycine, permeabilized with 1.5% (v/v) Triton X-100 in PBS for 30 min and then incubated in 0.3% Triton X-100, 1% (w/v) Bovine Serum Albumin (BSA), 10% (v/v) Normal Donkey Serum in PBS for 1 hour. Samples were incubated O/N at 4°C with primary antibodies in PBS, 0.3% Triton X-100, 1% Bovine Serum Albumin (BSA), and 2% Serum. The antibodies used were: mouse anti-α-actinin (1:700, Sigma); mouse anti-caveolin-3 (1:500, BD Biosciences); rabbit anti-HCN1, -HCN2 and -HCN4 (1:100, Alomone Labs); rabbit anti-β1 (1:50, Santa Cruz) and anti-β2 adrenergic receptors (1:50, Santa Cruz) and anti-muscarinic M2 receptors (1:200, Santa Cruz), chicken anti-GFP (1:1500, Abcam). Samples were then washed in PBS and incubated for 1 hour with the appropriate fluorophore-conjugated secondary antibodies (donkey anti-mouse Alexa488; donkey anti rabbit Alexa594, goat anti rabbit Alexa 405 1:500, Molecular Probes) in PBS, 0.3% Triton X-100, 1% Bovine Serum Albumin (BSA), and 2% Normal Donkey Serum. After a final washout in PBS, coverslips were mounted with Vectashield mounting medium with or without DAPI (Vector). Confocal images were acquired using a video confocal microscopy ViCo (Nikon). The proportion of cells expressing α-actinin and/or HCN4 was calculated as the ratio between the number of cells not labeled and the total number of nuclei visible in each image. For each time point, three or more fields from at least two different experiments were counted.

**BrdU proliferation assay**

CD166+ cells (24 and 48h after sorting) and ES cells, were incubated for 1h in culture medium with 50 μmol/L of 5-Bromo-2’-deoxyuridine (BrdU, Sigma) in a 5% CO2 incubator at 37°C. Cells were then washed twice in PBS and fixed in EtOH 95% and Acetic acid 5% for 20 min at RT. Cells were washed once with 1.5 mol/L HCL for 10 min then rinsed twice in PBS and finally permeabilized with 0.25% Triton X-100 in PBS for 5 min. Cells were incubated with the mouse anti-Bromo-deoxyuridine (clone BU-1, GE Healthcare) for 1h at 4°C. Samples were washed three times with PBS and once with 0.25% Triton X-100 for 5 min and incubated with the donkey anti-mouse Alexa488 secondary antibody for 40 min in the dark at RT. After a final washout in PBS, coverslips were mounted with Vectashield mounting medium with DAPI (Vector). Proliferation was evaluated by counting the proportion of BrdU-positive cells in several microscopy fields (> 9 fields from 3 different experiments).

**Supplementary Results**

**Pluripotent gene analysis and in vivo teratoma assay.**

One of the major drawbacks of the use of pluripotent stem cells resides in their high teratogenic potential. In order to avoid teratoma formation, it is essential to purify a committed population of cells with a limited proliferative potential and in which pluripotency genes have been turned off. To this aim, after selection, CD166+ cells were allowed to re-aggregate in suspension for 24 hours and then plated. CD166+ cells express negligible levels of the pluripotency genes Oct4, Nanog and Rex1 that were instead expressed at significantly higher levels in both CD166- and undifferentiated ESCs (Online Figure VIII A). Moreover, since CD166 expression is upregulated in several tumors, we have evaluated its expression in CD166-selected cells after 8 and 25 days in culture; as expected from maturing cardiac cells, CD166 was significantly downregulated with time (Online Figure VIII A).

Given the low yet present proliferative potential of CD166+ cells and given the fact that even few undifferentiated ESCs can generate a teratomas in vivo, we performed a teratogenic assay by subcutaneously inoculating either 5x10^5 matrigel-embedded CD166+ or ESCs, constitutively expressing nLacZ, (Online Figure VIII B-E), in CD1 nude mice. As expected, ESCs transplanted mice (n=3) developed a teratoma within two weeks from the injection (Online Figure VIII F-K) while those injected with CD166+ (n=4) did...
not develop any teratoma even when CD166+ cells were increased to $1.5 \times 10^6$. Mice injected with CD166+ cells have been followed-up for more than one year. These data confirm that CD166+ cells have lost the pluripotency and thus the teratogenic features typical of ESCs and suggest that CD166+ precursors may represent a suitable and safe cell source for in vivo applications.

**Online Figure legends**

**Online Figure I** CD166+ expression in extracardiac tissues. Confocal images obtained from slices of E10.5 (A,B) and E12.5 (C,D) embryos showing the expression of CD166 in extracardiac tissues/organs. Ltg, laryngo-tracheal groove; mg, mid gut; e, esophagus; reca, right common carotid artery; dta, descending thoracic aorta.

**Online Figure II. Selection of CD166+ cells from CGR8 ESC line.** Flow cytometry analysis of CD166-expressing cells derived from 8 day-old EBs obtained from ES-CGR8 line (Left). α-actinin (α-act) expression by qRT-PCR in CD166 positive and negative cells derived from the CGR8 cell line (right). * denotes p<0.05.

**Online Figure III. Comparison of ectodermal, endodermal and non-cardiac mesodermal gene expression in CD166-positive and negative cells.** Expression levels of synaptophysin (ectoderm) tranthyretin (endoderm) and MyoD (non-cardiac mesoderm) in CD166+ and CD166- cells. Brain, liver or skeletal (sk) muscle and fibroblasts or ventricles were used as positive and negative controls, respectively. * indicates p<0.05 versus the other groups.

**Online Figure IV. Expression of α-actinin and HCN4 in cultured CD166+ and CD166− cell aggregates.** Confocal images of sorted CD166+ and CD166− cells fixed after the day after re-aggregation (day2) and after the subsequent two days. Cell aggregates were co-stained with antibodies against the cardiac protein sarcomeric α-actinin and the pacemaker channel HCN4, a typical marker of sinoatrial cells 6–8. Nuclei were stained with DAPI. Calibration bars 50 μm.

**Online Figure V. SAN genes expression in CD166− cells.** Comparison in the expression of the typical SAN genes HCN4, HCN1, ssTnI and Shox2 by qRT-PCR between CD166− and CD166+ cells. * denotes p<0.05.

**Online Figure VI. Late CD166 cells do not show any enrichment of specific atrial or ventricular genes.** Comparison of the expression levels of the atrial marker Myh6 and of the ventricular marker mlc2v in late CD166 cells, SAN and either atria or ventricle. * denotes p<0.05 vs atria or ventricles.

**Online Figure VII. Comparison between CD166+ cells and native mouse SAN cells.** Confocal images of a 25 day-old CD166-selected cell aggregate and of a mouse SAN slice labeled with an antibody anti-HCN4 (red). Nuclei were stained with DAPI. Calibration bars 50 μm.

**Online Figure VIII. Analysis of pHCN4-EGFP EBs and corresponding CD166-selected cells.** A, confocal images of a 24 day-old pHCN4-EGFP EB showing overlapping expression of GFP (green), cav-3 (red) and HCN4 (blue). B, epifluorescence microscopy images of pHCN4-EGFP CD166-selected cells (left) together with the corresponding phase contrast field (right). Calibration bars 50 μm.

**Online Figure IX. CD166+ cells do not express pluripotency markers and are not teratogenic in vivo.** A, qRT-PCR analysis of the pluripotency genes, Oct4, Nanog and Rex1 and comparison of CD166 expression in CD166-selected cells after 8 and 25 days in culture. * denotes p<0.05 vs ES; # denotes p<0.05 vs CD166−; § denotes p<0.05 vs day 8. B, phase contrast image of single nLacZ ES cells stained by x-gal. C, flow cytometry analysis of nLacZ-ES cell isolated from day8 EBs showing that the proportion of CD166+ cells is similar to that of control (non-infected) ES cells. D,E, sorted CD166+ LacZ cells form a spontaneously beating syncytium (D and online video IV) positive to x-gal staining (E). F-K, hematoxylin and eosin and x-gal staining of the teratoma, providing examples of differentiation of injected mESC, into derivatives of the three germ layers: ectoderm (F,G, squamous epithelium), endoderm (H,I, cartilage) and
mesoderm (J,K. gut-like structure with mucinous epithelium and glands). X-gal staining was performed on sections adjacent to those used for H&E. Calibration bars 100 μm.

References


Online Figures

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Online Figure III
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Online Figure IV
Online Figure VI
Online Figure VII

CD166+ d25

mouse SAN
A  pHCN4-EGFP EBs (day 24)

GFP  cav3

HCN4  overlap

B  pHCN4-EGFP CD166-derived cells (day 8+10)

GFP  phase contrast

Online Figure VIII
Online Figure IX
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<td>NM_013697.4</td>
<td>catgaattcgcggatgtg</td>
<td>gatgggtgtaggtggcaggg</td>
</tr>
<tr>
<td>Myh6</td>
<td>Cardiac alpha-myosin heavy chain</td>
<td>NM_010856.4</td>
<td>tgcagagctcaagaaggt</td>
<td>cccagccatctctcttgta</td>
</tr>
<tr>
<td>Mlc2v</td>
<td>myosin light chain 2v</td>
<td>NM_010861.3</td>
<td>ggacacatttctgcccta</td>
<td>atcgtgaggaacggtga</td>
</tr>
</tbody>
</table>

**Online Table I.** Complete description of the gene analyzed, accession number and sequences of the primers pairs used.
Online Table II. Percentage of CD166+ and CD166− cells expressing α-actinin or HCN4. Numbers into brackets represents the number of fields in which cells have been counted.

<table>
<thead>
<tr>
<th></th>
<th>α-actinin</th>
<th>HCN4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD166+</td>
<td>CD166−</td>
</tr>
<tr>
<td>Day2</td>
<td>77.2±6.3% (3)</td>
<td>15.6±1.3% (4)</td>
</tr>
<tr>
<td>Day3</td>
<td>84.6±2% (4)</td>
<td>16.7±1.6% (6)</td>
</tr>
<tr>
<td>Day4</td>
<td>87.6±1.7% (11)</td>
<td>17.3±7.4% (6)</td>
</tr>
</tbody>
</table>

**Online Video I. CD166+ cell aggregates 24 hours after sorting.** The video shows spontaneously beating CD166+ cell clusters plated after 24 hours of reaggregation in low adhesion dishes, at different magnifications.

**Online Video II. CD166-selected cells 21 days after sorting.** The video shows a spontaneously and vigorously beating CD166-selected cellular syncytium 21 days after sorting.

**Online Video III. pHCN4-EGFP CD166-selected cells 10 days after sorting** The video shows a spontaneously beating CD166-selected cellular syncytium obtained from engineered ES cells expressing the EGFP under the transcriptional control of the HCN4 gene promoter.

**Online Video IV. nLacZ CD166-selected cells 10 days after sorting.** The video shows a spontaneously beating CD166-selected cellular cluster obtained from engineered ES cells constitutively expressing the nLacZ gene.