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

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ABSTRACT

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 to use CD166 expression for isolating SAN progenitors from differentiating embryonic stem cells (ESCs).

Methods and Results: We found that in E10.5 mouse hearts CD166 and HCN4, a marker of the pacemaker tissue, are co-expressed. Sorting ESCs 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 increase (+57%) and acetylcholine decreases (-23%) the beating rate of CD166-selected cells which indeed express the β-adrenergic and muscarinic receptors. In co-cultures, 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 non-teratogenic population of cardiac precursors able to mature and form a fully functional SAN-like tissue.

Keywords:
Cardiac progenitor cells, pacemaker, sinoatrial node, embryonic stem cells, HCN channels.

Nonstandard Abbreviations and Acronyms:

SAN	sinoatrial node
iPSC	induced pluripotent stem cells
EBs	embryoid bodies
Iso	Isoproterenol
Ach	acetylcholine
BrdU	Bromodeoxyuridine
nLacZ	nuclear LacZ
cav3	caveolin-3

INTRODUCTION

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 last two decades, the increasing implantation rate of electronic pacemakers has been primarily due to isolated sinus node dysfunction.[1] With the increasing population ageing, dysfunctions of the conduction tissue, which may trigger threatening arrhythmias, are expected to become more and more common.

With the advent of gene- and cell-based therapeutic approaches, researchers have focused their efforts to 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, iPSC).[4-7] Unfortunately, the high self-renewal capacity and plasticity of pluripotent stem cells, which make them interesting for regenerative purposes, represent their greatest disadvantages mainly because these features imply a high teratogenic potential. There is evidence though that in vitro cell commitment/differentiation of ESCs, would eliminate the risk of teratoma formation.[5,8]

A limiting step in sight of developing therapeutic applications using pluripotent stem cells consists in the isolation of a homogeneous population of cells with the desired phenotype. We and others have for example shown that mouse ESCs differentiate into cardiomyocytes with the molecular and functional features of mature pacemaker cells; these cells are nonetheless scarce in number and interspersed with other cell types. [4,9] 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 hardly suitable for future clinical applications. Alternative selection methods are hampered by the lack of specific extracellular cardiac markers exploitable for cell sorting. There are however 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, at a specific differentiation stage, precursors of pacemaker cardiomyocyte.

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 employed 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 both by the local Institutional Review Board and by the Italian Ministry of Health.

ESCs culture and differentiation.
Mouse ESCs (D3 line, ATCC, and CGR8 line) were grown and differentiated as embryoid bodies (EBs) as previously described[4].

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's instruction. Analyses were carried out either soon after the sorting procedure or after 24h of cell re-aggregation.

Quantitative (q)RT-PCR.
Gene expression was quantified by qRT-PCR (Line-GeneK, Bioer) using SYBER® Premix Ex Taq™ II (Takara), 50 ng of cDNA and 500 nmol/L of primers (for primers see online Table I). Data are expressed as $2^{-\Delta Ct}$ x 100. Due to the large range of values among the populations, statistical analysis was performed on the logarithm of $2^{-\Delta Ct}$ x 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 (Iso) and acetylcholine (ACh) 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 as previously described.[4]

Immunofluorescence and video-confocal analysis.
Samples were fixed in 4%paraformaldehyde, permeabilized and incubated O/N 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 LSD mean comparison or Student's test for independent populations were used as appropriate. Significance level was set to p=0.05.

RESULTS

CD166 is co-expressed with HCN4 during heart development.

So far, CD166 is the only marker detected also in the sinus venosus, 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, developing hearts of E10.5 mouse embryos, CD166 and HCN4 signals are almost completely overlapped (Figure 1 A-C). At E12.5, HCN4 and CD166 still co-localize in the region corresponding to the developing SAN (Figure 1 D-F), but as previously reported,[11] CD166 expression broadens and becomes more evident in ventricles (Figure 1E) and in several extracardiac organs/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 mESC.

We carried out 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, in order 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 the CD166-negative (CD166–,P2) and CD166-positive (CD166+, P3) subpopulations present at day 6, 8, 10 and 15 of differentiation. The CD166+ population was 1.4 ± 1.3% at day 6 and increased to 12.1±6.4%, 20.7±8.1% and 37.3±9.0% at days 8, 10 and 15, respectively. Following cell sorting, the qRT-PCR revealed significantly higher levels of α-actinin in the CD166+ than in the CD166– populations at day 6 and day 8, while at day 10 and 15 α-actinin expression in CD166+ and CD166– was similarly low, indicating the loss of cardiac specificity (Figure 2B). We therefore chose day 8 for selection of CD166+ cells, in order to optimize at one time the yield and cardiac specificity.

To evaluate the purity of the sorting procedure, we compared the expression levels of CD166 in the two populations and in the undifferentiated ESCs; as expected only the CD166+ population showed a high level of expression (Figure 2C). Other cardiac markers such as cTnI, Mef2c and GATA4 were significantly more expressed in CD166+ cells than in CD166– population or in ESCs (Figure 2D).

Since it is known that the cardiac differentiation potential may differ from clone to clone and among different cell lines,[16] we repeated the selection using a different ESC line (CGR8). Flow cytometry and qRT-PCR analyses on CGR8 ESCs confirmed that at day 8, α-actinin expression in CD166+ cells was 10-fold higher than in CD166– cells (Online Figure II), indicating that our selection procedure is effective in isolating cardiac precursors, independently from the ESCs line used.

To better characterize the CD166 population we carried out a FACS analysis on cells dissociated from 8 day-old EBs, to check for the expression of markers typically expressed in cardiovascular precursors (flk-1, Sca-1 and c-kit), in mesenchymal stem cells (CD44 and CD90), and in hematopoietic precursors (CD34).[17-20] Representative dot plots of Figure 3 show that although flk-1, Sca-1 and c-kit are expressed in 8 day-old EBs (Q1), the fraction of cells co-expressing one of these markers with CD166 (Q2) is very low. The same is true for the mesenchymal markers CD90 while CD44 was expressed in a small proportion of CD166+ cells. As expected, the hematopoietic marker CD34 was not expressed at all. On average, the fraction of double positive cells was: flk-1+/CD166+, 0.3±0.1%; Sca-1+/CD166+, 0.6±0.1%, c-kit+/CD166+, 3.7±2.8%; CD90/CD166+, 1.0± 0.8%; CD44/CD166+, 6.5±2.3%; CD34/CD166+, 0% (n=3).

We also evaluated the expression of genes whose expression is specifically associated with the endodermal, ectodermal and noncardiac mesodermal lineages in CD166+ cells. 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 sub-population of cardiac precursors.

CD166+ cells express typical SAN genes.

After sorting, and 24h of re-aggregation, most of the CD166+-derived aggregates started to beat spontaneously (see online Video I), and continued to beat vigorously in culture (see online video II) for up to four weeks. Spontaneous contraction was never seen in aggregates derived from CD166– cells. We quantified the fraction of cells expressing α-actinin and/or HCN4 at various days after sorting (2, 3 and 4, Online Figure IV). α-actinin was expressed in the 77-87% of the CD166+ and in the 15-17% of the CD166- cells while HCN4 was expressed in 82-84% of the CD166+ and 1-16% of CD166- cells (see Online Table II for actual values).

We then compared, by qRT-PCR, the mRNA levels of several genes either expressed in the embryonic and adult SAN or in ventricles with the levels found in early (just after sorting) or late (3-4 weeks in culture) CD166+ cells (Figure 4). We first analyzed the expression of the transcription factors Tbx18, Tbx3, Isl1 and Shox2, which are important in SAN formation.[21-23] The expression of these genes was high in early CD166+ cells and, even though it decreased in late cultures, remained at levels comparable with those found in the SAN and 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, CaV1.3, Cx30.2) are expressed at significantly higher levels than in the ventricle. Expression of the T-type calcium channel CaV3.2, high in early CD166+ cells, decreased significantly at later stages; nevertheless, this decrease was accompanied by a slight increase of the CaV3.1 isoform the other T-type calcium isoform expressed in the SAN;[24,25] a similar isoform switch between CaV3.2 and CaV3.1 has been previously documented during both mouse development and mESCs differentiation.[26,27] Connexin 45 (Cx45), an isoform found in all cardiac regions of the embryonic heart, and down-regulated after birth,[24,28] was expressed at similar levels in all groups but in early CD166+ cells where its expression was significantly higher. We finally quantified in CD166+ cells, 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 have also 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.

Since HCN channels are critical for pacemaker activity in both embryonic and adult SAN,[29,30] and 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 D) while HCN2, the main ventricular isoform,[32] could not be detected in α-act-positive cells (Figure 5G,H). Panel I of Figure 5 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 also a morphology similar to that of adult SAN myocytes (Figure 5J,K and M,N) and, like adult SAN cells, showed co-localization of HCN4 and cav3 staining (Figure 5L).[33] Furthermore, like SAN, CD166-selected cells expressed negligible levels of the atrial-specific alpha-myosin heavy chain (Myh6) and of the ventricular-specific myosin light chain 2v (mlc2v), suggesting no progression of differentiation towards a chamber phenotype (Online Figure VI).

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

Since CD166+ cells express the HCN isoforms (HCN4 and 1), typically found in the SAN, we also analyzed the I_f current. CD166-selected cells display the I_f current both after 1 (Figure 6A left) and 3 weeks in culture with a voltage dependence comparable to that of native mouse SAN cells (Figure 6 A 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; see also ref 30).

We evaluated also the contribution of the calcium currents ICaL and ICaT to spontaneous activity of CD166-selected cells by superfusing the ICaT blocker Ni^{2+} (50 μM) and the ICaL blocker nifedipine (0.1 μM) during spontaneous AP recordings (Figure 6B); on average, Ni^{2+} decreased rate by 17.7±2.6% (n=5) and nifedipine by 16.9±6.4% (n=3); these values were not significantly different from those obtained from mouse SAN cells (Ni^{2+} 21.6±7.5,n=5; nifedipine 16.5±2.2, n=6)

As mentioned earlier, CD166-selected cardiomyocytes maintain their spontaneous activity for more than 3 weeks in culture; during this period, their beating rate increased (Figure 7A) and the increase was comparable to that of normal mouse embryonic development,[34] suggesting a certain degree of maturation.
We finally 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-, β2-adrenergic or anti-M2 muscarinic (M2 Ach) 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 (Iso, 1 μmol/L) or the muscarinic agonist acetylcholine (ACh, 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 EGFP under the transcriptional control of the HCN4 promoter (pHCN4-EGFP, see online Methods). pHCN4-EGFP-derived EBs displayed EGFP-positive contracting portions co-expressing caveolin 3 and HCN4 (Online Figure VIII A). As expected, when

CD166+ cells were selected from pHCN4-EGFP EBs, the whole beating layer (online video III) showed the EGFP signal (Online Figure VIII B).

CD166-selected cells drive the rate of co-cultured neonatal ventricular myocytes.

We have then evaluated if CD166-selected cells can functions as a pacemaker; to this aim we have employed a widely used co-culture system.[35-37] Rat neonatal ventricular myocytes were plated on top of both, spontaneously beating CD166+ cells or of quiescent CD166–, or were plated alone. After a few days, when a syncytium was formed, spontaneous rate was calculated from action potentials recordings. Cardiomyocytes in co-culture with CD166+ cells had a mean rate of 1.7±0.19 Hz (n=5, data not shown), significantly higher than the rate obtained from both cardiomyocytes in co-culture with CD166– (0.82±017 Hz, n=6) and from cardiomyocytes alone (0.84 ±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 BrdU staining; CD166+ cells showed a low proliferative potential at 24h (7.2 ± 2.7%), a value that further decreased at 48h (3.8±1.8%); as expected, the BrdU 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 (see supplementary material and Online Figure IX for details).

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 in sight of the development of either a cell-based therapeutic approach aimed at re-establishing 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 since they can generate spontaneously beating cells with the molecular and functional features typical of SAN/pacemaker myocytes.[4,6,7,38]

Since pacemaker cells originating from differentiating ESCs are interspersed among other cell types,[4] their specific selection and isolation remains a major challenge.

So far, two markers have been found to be expressed both in the heart and in ESC-derived cardiomyocytes: CD166 (or ALCAM)[11-13,39] and CD172a (or SIRPA).[40] The fact that SIRPA is expressed in both the fetal and adult human heart (in both atria and ventricles), and that in hESCs, can be used to select a subpopulation enriched in cardiac troponinT-positive precursors, rules against its use as a selection marker for pacemaker precursors. Furthermore, SIRPA could not be detected in mouse heart,[40] suggesting that it does not represent an evolutionary conserved protein for cardiac development.

We have instead 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 indeed shown that CD166 expressing cells express also HCN4 (Figure 1), a protein that specifically delineates the SAN region both during embryogenesis[14,15,41] and in adulthood.[42-45] HCN4 is also fundamental for sinus node function since its absence is incompatible with life[29,30] and mutations in its sequence can cause inherited rhythm disturbances.[46]

Here we show that freshly selected CD166+, but not CD166– cells, express high levels of HCN4 mRNA and already 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 cell agglomerates like those of native SAN (Figure 5J-N and online Figure VI).

CD166+ cells express also high levels of Tbx18, Shox2, Tbx3 and Isl-1 which are known to characterize the pool of mesodermal progenitors which separate early during cardiogenesis[15] to originate first 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.[47] After three weeks in culture, the expression level of these genes is still comparable to that found in the SAN, and higher than those found in the ventricle. Our data thus 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 sack.[12] At embryonic day 8.5, yolk sack-derived CD166+ cells originate both endothelial and cardiac cells, however if CD166+ cells are selected also for flk-1, only the CD166+/flk-1□ population formed cardiomyocytes.[12] In agreement with this evidence, our data show a negligible co-expression of CD166 and flk-1.

Other data show however that ESC-derived flk-1+ cardiac precursor express also c-kit and sca-1, [17,48] and that a subpopulation of ESC-derived selected by sca-1 formed α-MHC-positive contracting aggregates.[49] CD166+ cardiac precursors do not express significant level of either flk-1 or Sca-1 and 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*.[13] have shown that CD166 selection of differentiating human ESCs results in a population enriched in cells defined as embryonic cardiomyocytes on the base of ion channels and structural proteins expression. We have instead found that mESC-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*.[13] could arise both 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 about 20-30% while our CD166-sorted cells have a much lower proliferation rate (<4% at 48 hrs from sorting). Moreover their cells maintain a high CD166 expression for at least two weeks while in our cells CD166 expression decreased significantly (Online Figure VIII A), as expected during cardiomyocytes maturation.[11]

Previous attempts to generate pacemaker/SAN myocytes from ESCs have used either a cell engineering[9] or a pharmacological approach[5,50] but neither one resulted in a homogeneous population. Morikawa *et al*.[9] generated mESCs expressing the EGFP under the transcriptional control of HCN4 gene promoter. Contracting areas of these engineered EBs expressed EGFP, HCN4 and other cardiac proteins but when EGFP+ cells were sorted, the majority did not show spontaneous action potentials but instead expressed the neuronal marker nestin,[9] which is in line with the fact that neuronal cells also express HCN4 channels.[14] Pharmacological treatment of differentiating ESCs with either 1-ethyl-2-benzimidazolinone (EBIO) or with suramin increased the fraction of sinus node-like cell population by 6 and 3 folds, respectively however these treatments did not prevented contamination by other cell lineages.[5,50]

In addition to the gene expression pattern, our CD166-selected cardiomyocytes also display the functional properties peculiar of the SAN cardiomyocytes. The main function of SAN myocytes is to spontaneously generate action potentials and to modulate cardiac rhythm so as to account for changes in oxygen demands. CD166-selected cardiomyocytes form a synchronously beating syncytium (see online videos II and III) which fires repetitive and regular action potentials. It is interesting to note that CD166-selected cardiomyocytes, like embryonic hearts,[34] increase their beating frequency with time, reaching a rate similar to that previously reported for dissociated adult mouse SAN myocytes.[30,51] 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 I_f

and the effect on rate exerted by the blockade of calcium channels are the same in freshly isolated sinoatrial mayocytes 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/differentiated prior of injection.[8] In this respect, 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 form a spontaneously beating syncytium and are able to electrically connect to and pace a monolayer of ventricular myocytes in vitro, their ability 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 to translate the present protocol to hiPSC, even though CD166 expression has been already reported in the human heart and in human ESC- and iPSC-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 in the embryo will originate the sinus node.[21,22] We can thus 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, Shox-2 and Tbx3 preventing the switch-on of gene programs leading to the working myocardium.[22,23] The CD166-selected cells thus 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 both for clinical and for in vitro pharmacological applications.

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DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1. CD166+ expression in the developing cardiac conduction system. A,D, Hematoxilin & eosin staining of E10.5 (A) and E12.5 hearts; ra, right atrium; la, left atrium, rv, right ventricle; lv, left ventricle; avc, atrioventricular canal; rsh right sinus horn; rscv, right superior caval vein. **B,C,E,F,** confocal images of adjacent slices of E10.5 (B,C) and E12.5 heart (E,F) showing the expression of CD166 and HCN4 proteins.

Figure 2. CD166 recognize cardiac precursors. **A**, Representative dot-plots of CD166– and CD166+ populations (P2 and P3 gates respectively) obtained by flow cytometry analysis of cells dissociated from EBs (P1 gate); at different time points of differentiation. **B**, qRT-PCR analysis of cardiac α-actinin (α-act) in CD166+ (white bars) and CD166– (gray bars) at the various times. **C, D**, quantitative expression analysis of CD166 (C) and of the cardiac genes, cTnI, Mef2c and GATA4 (D) in unsorted ESCs and in CD166+ and CD166– cells sorted at day 8 of differentiation. * indicates $p<0.05$ *v* CD166+.

Figure 3. Flow cytometry characterization of the CD166+ population. Representative dot plots showing the proportion of CD166+ cells co-expressing cardiovascular (Flk-1, Sca-1 and c-kit), mesenchymal (CD90 and CD44) and hematopoietic (CD34) markers at day 8 of differentiation.

Figure 4. Comparison of gene expression in early and late CD166+ cells, SAN and ventricle. qRT-PCR analysis of transcription factors (Tbx18 Tbx3, Isl1 and Shox2), structural proteins and ion channel (ssTnI, 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). * denotes $p<0.05$ vs SAN; # denotes $p<0.05$ vs ventricle.

Figure 5. HCN channel expression in CD166-selected cells. Confocal images of CD166-selected cells, after 1 (**A**-**H**) or 3 (**J**-**L**) weeks in culture, labeled with either the cardiac proteins α-actinin (α-act, green) or caveolin3 (cav-3, green) and the various HCN isoforms (red). Neonatal ventricular myocytes were used as positive control for HCN2 staining (**I**, red). Nuclei were stained with DAPI. **M,N,** Phase contrast images of a single CD166-derived cells 25 days after cell sorting (**M**), and of an acutely dissociated mouse SAN cell (**N**). Calibration bars 50 μm.

Figure 6. Functional comparison between CD166-selected cells and SAN myocytes. A left, representative I_f traces recorded from single CD166-selected cells after 1 week in culture ; **right,** mean activation curves obtained from CD166-selected cells after 1 (filled circles) and 3 weeks (open circles) in cultures and from mouse SAN myocytes (open squares); lines represent the best fitting by the Boltzmann equation. **B**, Representative action potentials recorded from CD166-selected cells (top panels) and SAN cells (bottom panels) in control (Tyrode) and during superfusion of either 50 μmol/L Ni^{2+} or 100 nmol/L nifedipine.

Figure 7. Autorhythmic activity of CD166+-derived cells and autonomic modulation. A, Mean rate calculated from spontaneously beating CD166-selected cells at different time points in culture; insets show representative action potential traces. **B**, confocal images showing co-expression of caveolin3 with β1 and β2-adrenergic receptors and of α-actinin with M2 muscarinic receptors. Nuclei were stained with DAPI. Calibration bars 50 μm. **C**, spontaneous action potentials recorded in control and during perfusion of 1 μmol/L isoproterenol (Iso) and 0.1 μmol/L acetylcholine (Ach) from CD166-selected cells; the bar graph shows the mean rate changes caused by the autonomic agonists.

Novelty and Significance

What Is Known?

- Embryonic stem cells (ESCs) may differentiate into cardiomyocytes with nodal-, atrial-, or ventricular-like properties.
- Selection of ESC-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 co-expressed with the HCN4 subunit of the pacemaker channels, the best known marker of the sinus node (SAN).
- Cultured CD166+ cells, selected from differentiating mouse ESCs, 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 CD166-expressing cells, which allows the recognition and isolation of SAN precursors from ESC. CD166+ cells express several genes involved in SAN development and present the typical molecular and electrophysiological properties of mature SAN cardiomyocytes. CD166-selected cells are also 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.

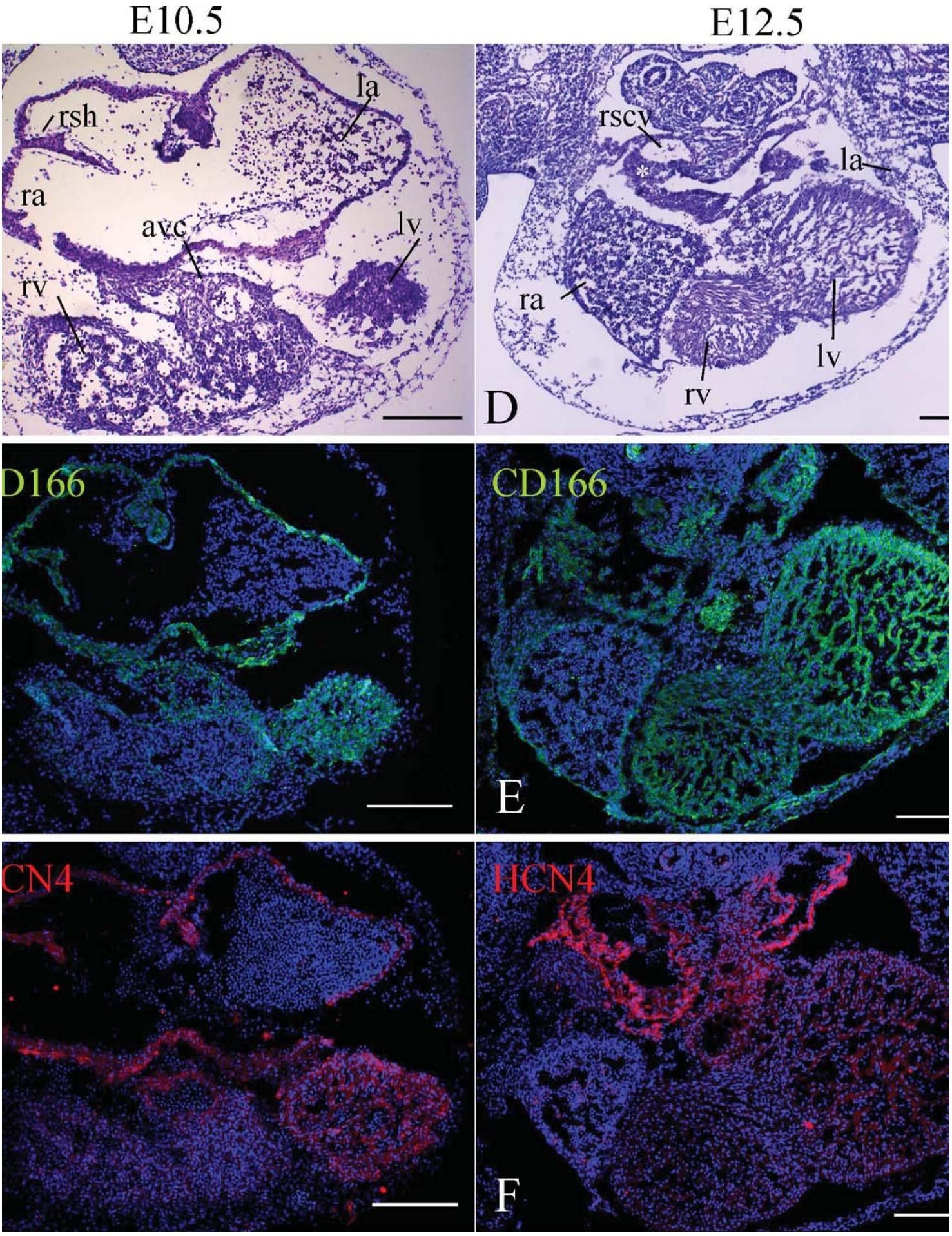
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Figure 2

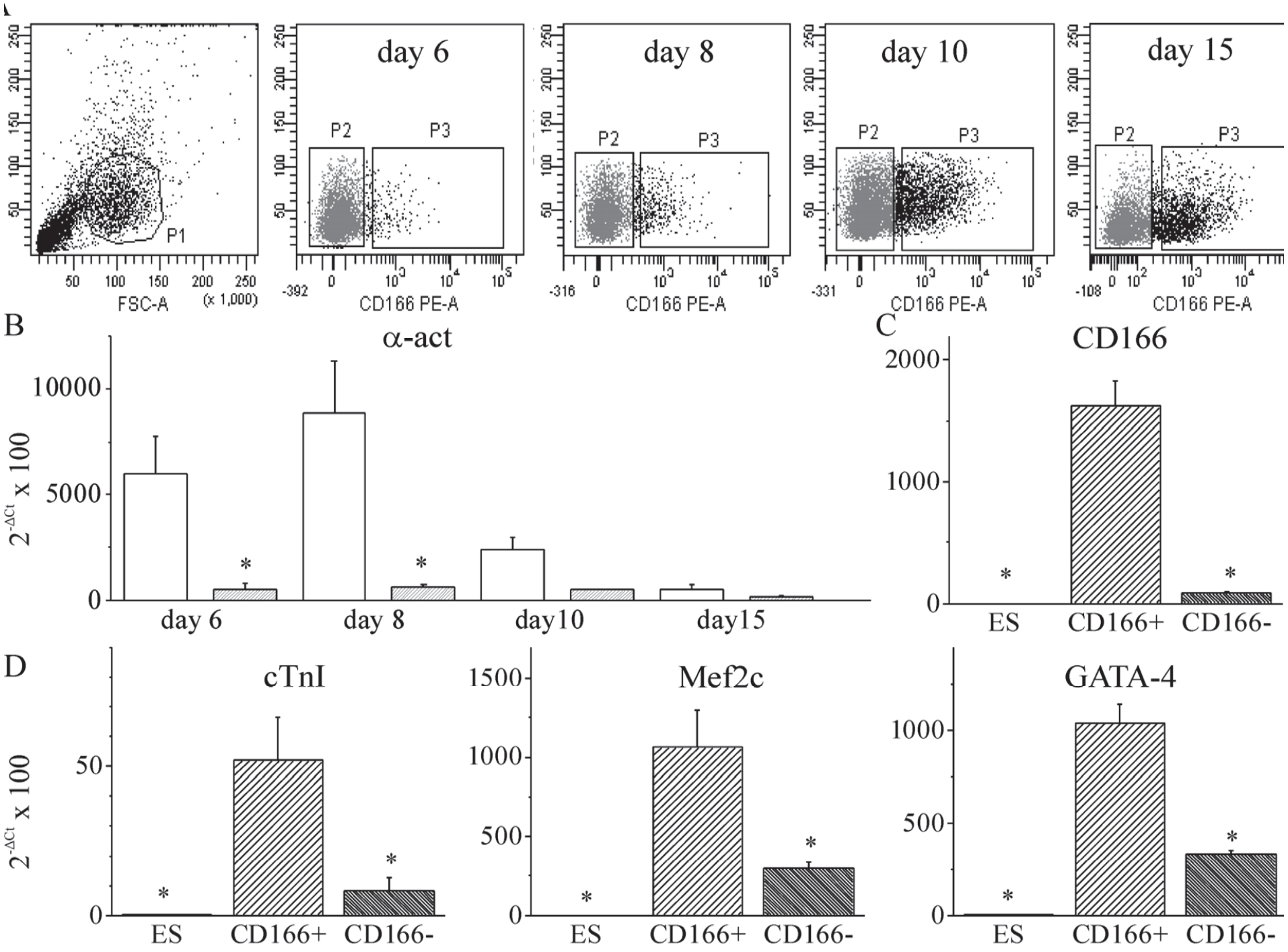
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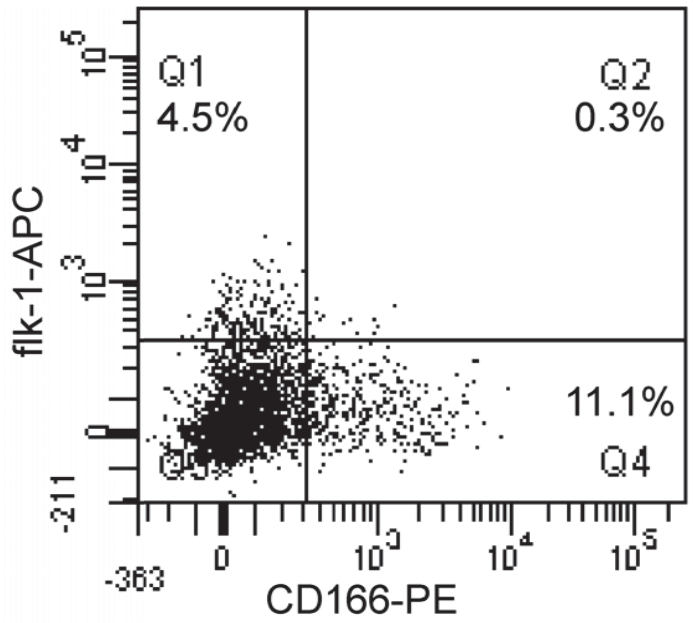
Figure 3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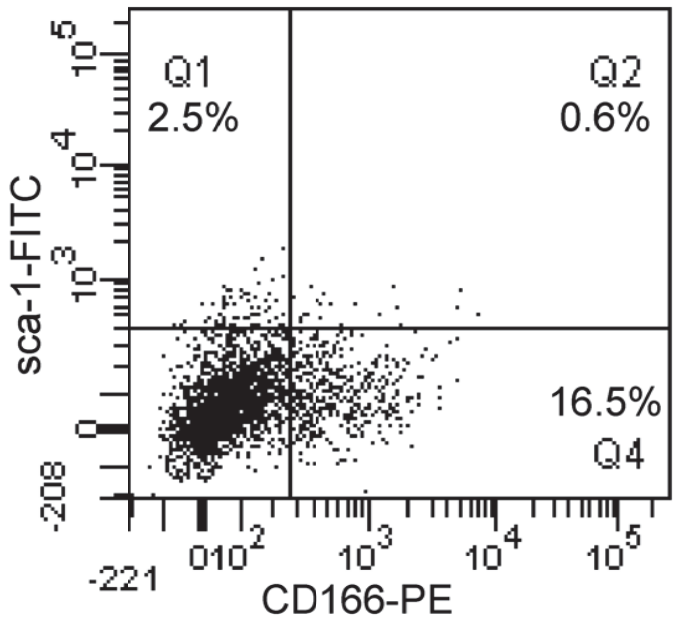
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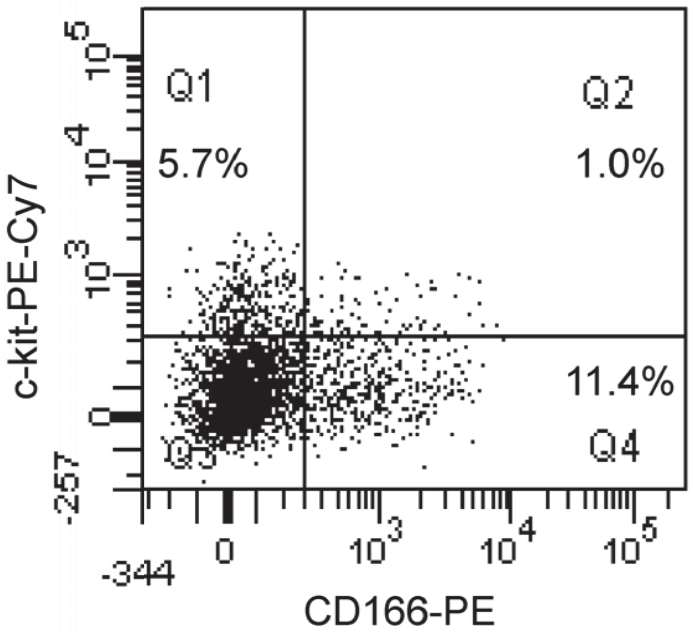
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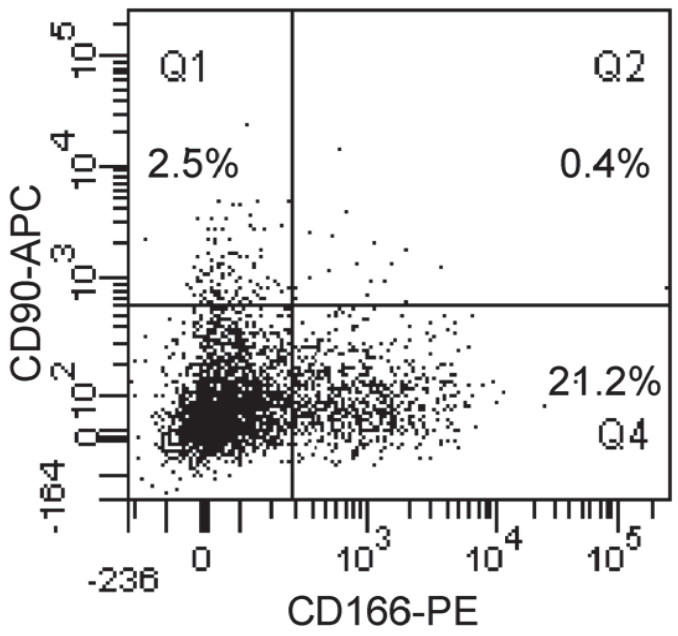
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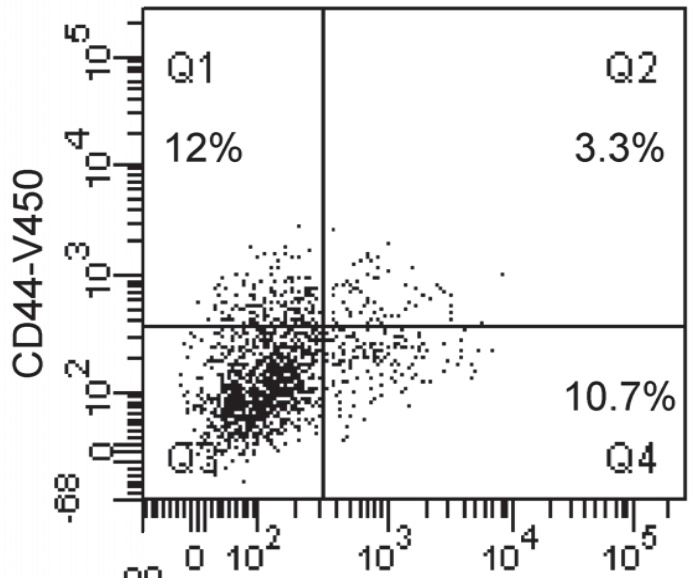
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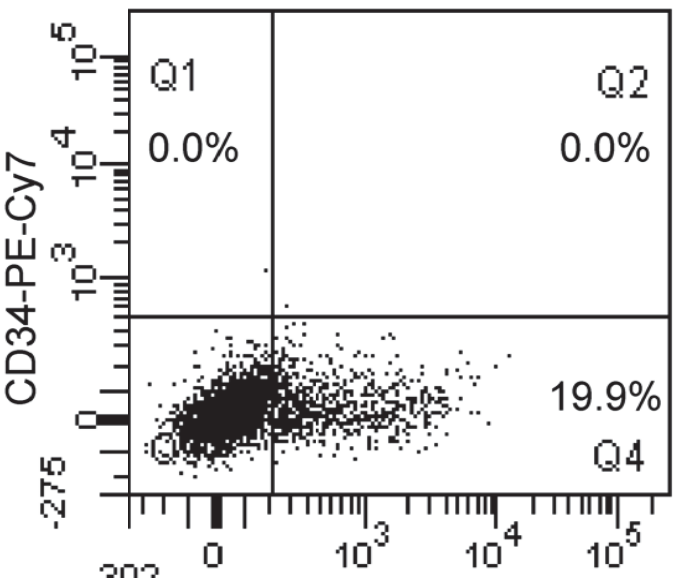
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Figure 4

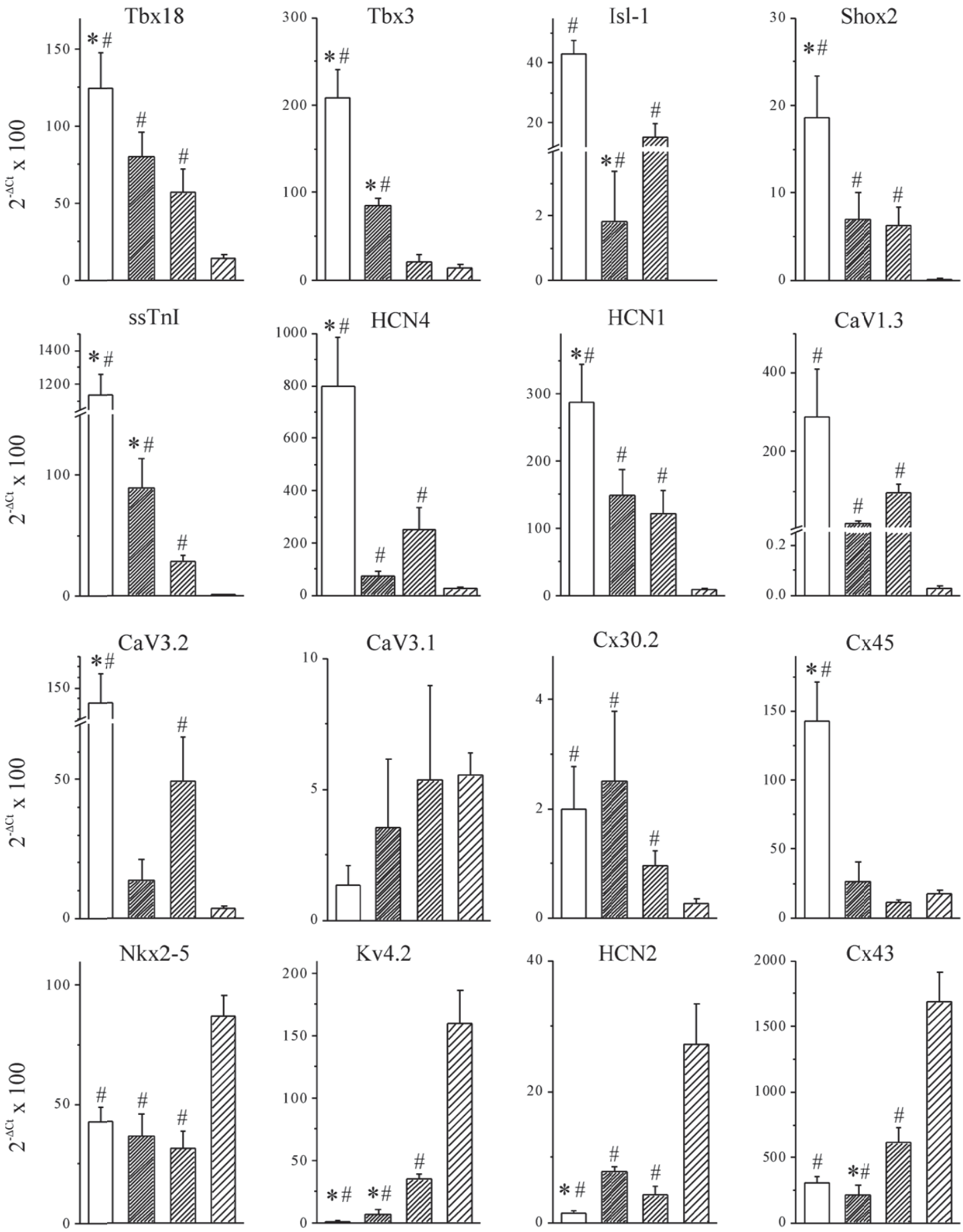
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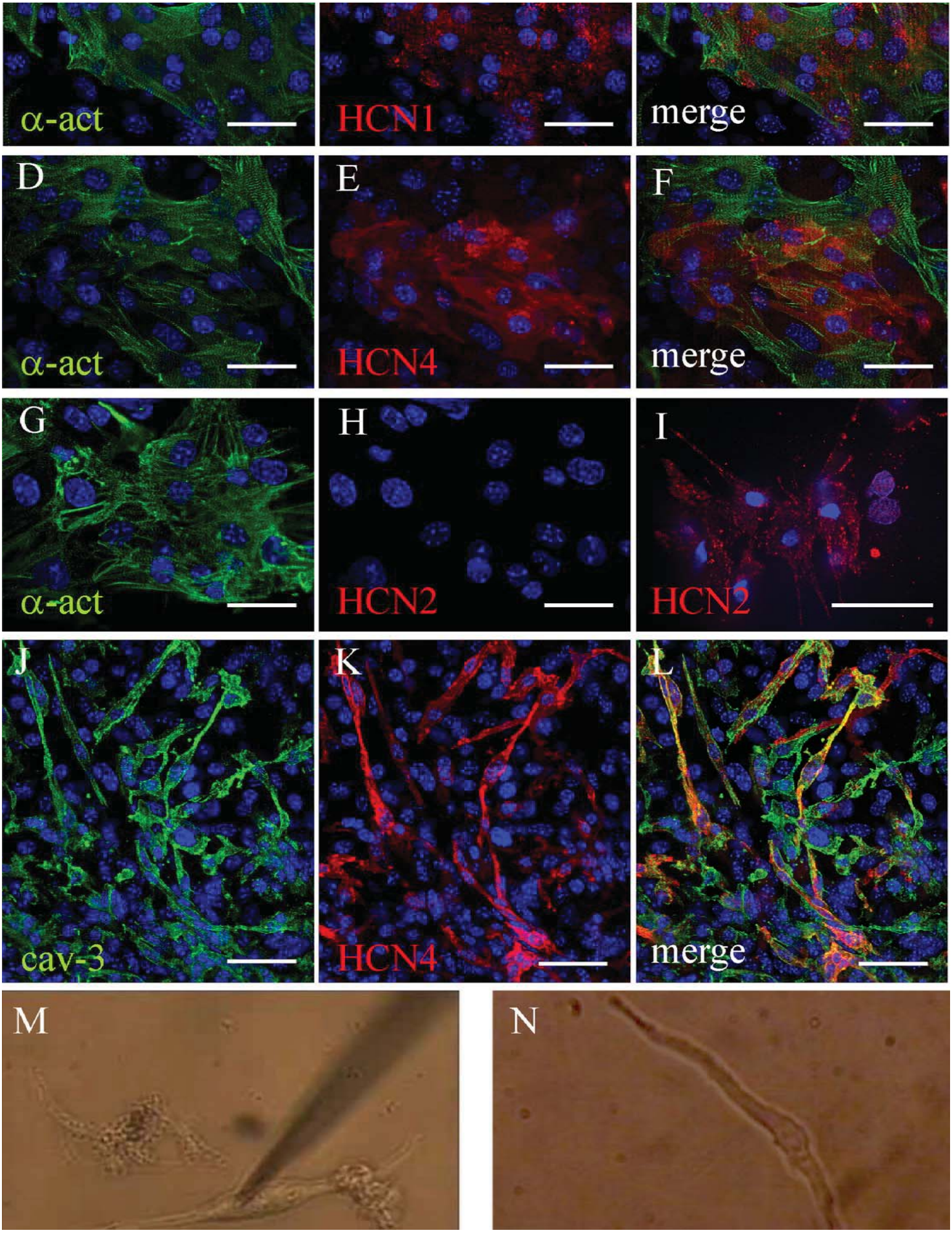
Figure 5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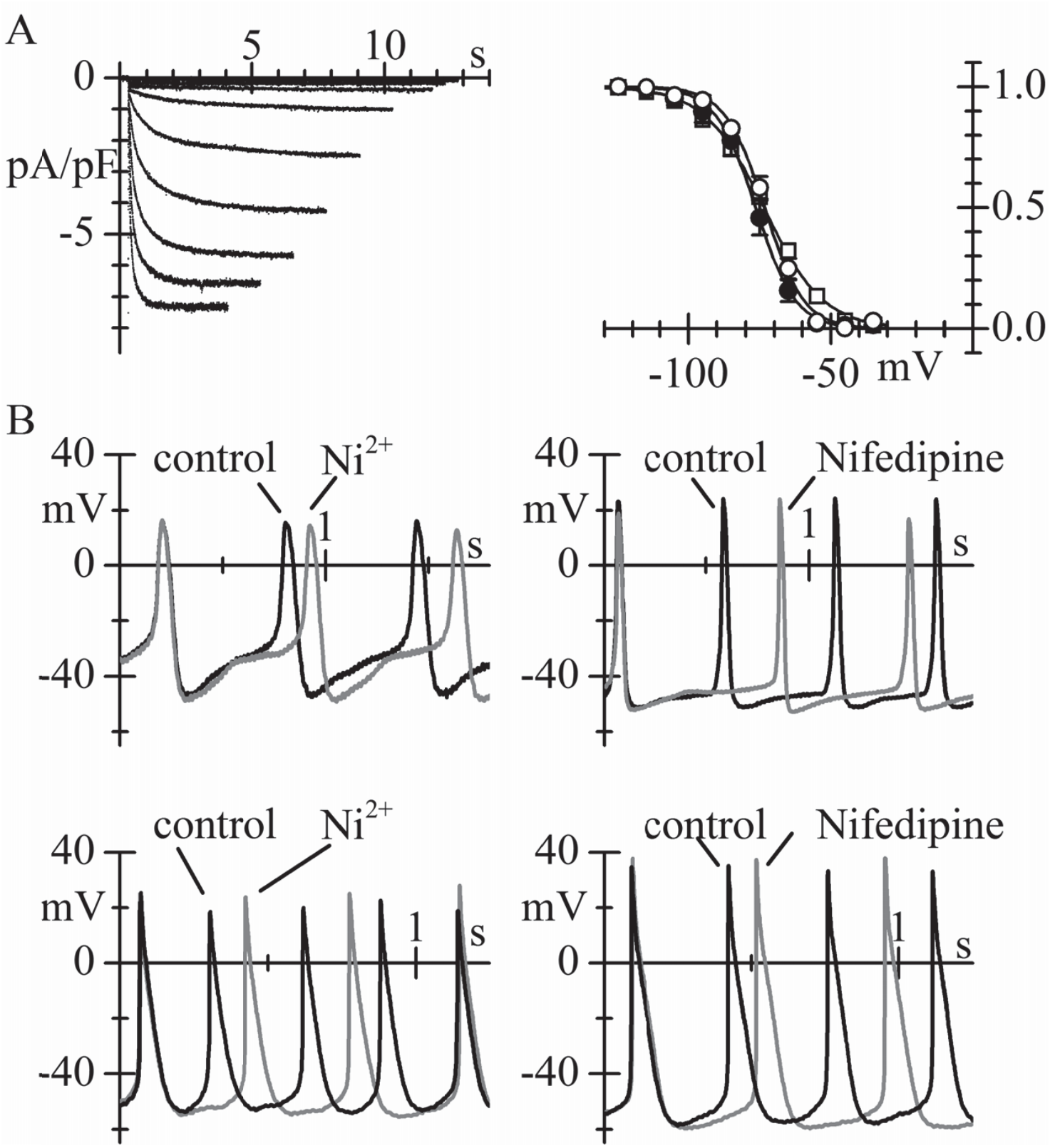
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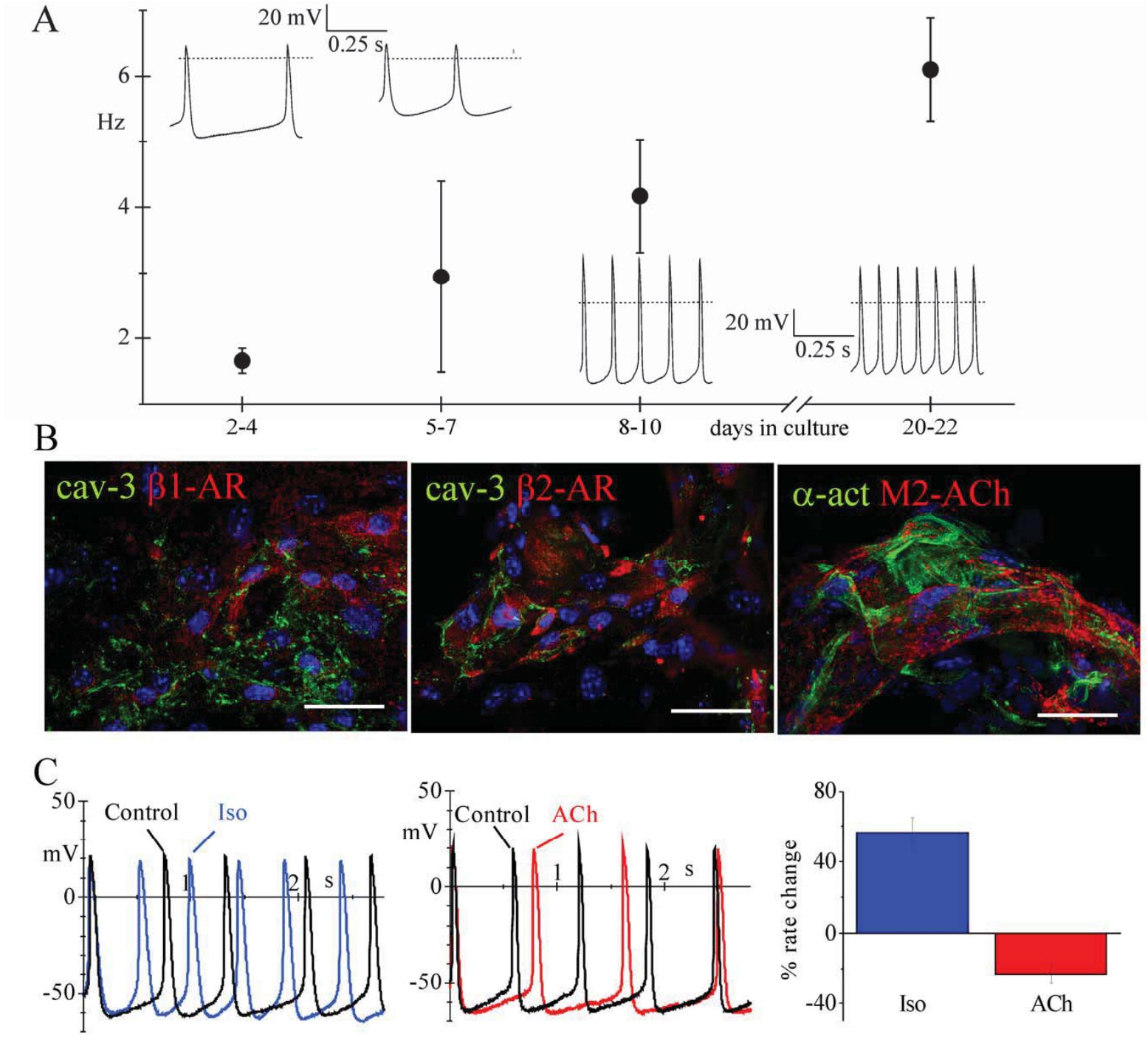
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