Role of $A_{2B}$ adenosine receptors in regulation of paracrine functions of stem cell antigen (Sca)-1 positive cardiac stromal cells.

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A\textsubscript{2B}KO, A\textsubscript{2B} adenosine receptor knockout; ADA, adenosine deaminase; APC, allophycocyanin; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; EBM, endothelial basal medium; EGM, endothelial growth medium; FAM, 5’-carboxyfluorescein; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HG, high glucose; IBMX, 3-isobutyl-1-methylxanthine; IFN\textgamma, interferon \gamma; IL, interleukin; LG, low glucose; mFGF-2, mouse basic fibroblast growth factor; NECA, 5’-N-ethylcarboxamidoadenosine; Sca-1, stem cell antigen-1; PBS, phosphate-buffered saline; PE, phycoerythrin; PSB-603, 8-[4-[4-(
chlorophenzyl)piperazide-1-sulfonyl)phenyl]-1-propylxanthine; RT-PCR, reverse transcription-polymerase chain reaction; SCH58261, 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine; TGF-β1, transforming growth factor β1; VEGF, vascular endothelial growth factor; WT, wild type

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ABSTRACT

Existence of multipotent cardiac stromal cells expressing stem cell antigen (Sca)-1 has been reported and their proangiogenic properties demonstrated in myocardial infarction models. In this study, we tested the hypothesis that stimulation of adenosine receptors on cardiac Sca-1+ cells upregulates their secretion of proangiogenic factors. We found that Sca-1 is expressed in subsets of mouse cardiac stromal CD31- and endothelial CD31+ cells. Population of Sca-1+CD31+ endothelial cells was significantly reduced whereas population of Sca-1+CD31- stromal cells was increased one week after myocardial infarction indicating their relative functional importance in this pathophysiological process. An increase in adenosine levels in adenosine deaminase-deficient mice in vivo significantly augmented VEGF production in cardiac Sca-1+CD31- stromal but not in Sca-1+CD31+ endothelial cells. We found that mouse cardiac Sca-1+CD31- stromal cells predominantly express mRNA encoding A2B adenosine receptors. Stimulation of adenosine receptors significantly increased IL-6, CXCL1 (a mouse ortholog of human IL-8) and VEGF release from these cells. Using conditionally immortalized Sca-1+CD31- stromal cells obtained from wild-type and A2B receptor knockout mouse hearts, we demonstrated that A2B receptors are essential for adenosine-dependent upregulation of their paracrine functions. We found that the human heart also harbors a population of stromal cells similar to the mouse cardiac Sca-1+CD31- stromal cells that increase release of IL-6, IL-8 and VEGF in response to A2B receptor stimulation. Thus, our study identified A2B adenosine receptors on cardiac stromal cells as potential targets for upregulation of proangiogenic factors in the ischemic heart.
INTRODUCTION

Recent studies have identified populations of cardiac resident cells that can be induced in vitro to transdifferentiate into different cell lineages (Matsuura, et al., 2004; Messina, et al., 2004; Beltrami, et al., 2003; Tateishi, et al., 2007; Pfister, et al., 2005; Wang, et al., 2006; Liang, et al., 2010; Matsuura, et al., 2009; Huang, et al., 2011). It has been suggested that these mesenchymal stem-like cells may play the role of resident cardiac progenitor cells. Indeed, the delivery of multipotent cell populations to injured heart resulted in improving neovascularization and attenuated the decline of cardiac function in animal models of myocardial infarction (Messina, et al., 2004; Tateishi, et al., 2007; Wang, et al., 2006; Martin, et al., 2008; Matsuura, et al., 2009; Huang, et al., 2011). However, the early assumption that these cells can replace damaged cardiomyocytes has recently given way to the realization that they also, and perhaps mainly, exert a beneficial effect via the release of paracrine factors including proangiogenic factors (Kinnaird, et al., 2004; Takahashi, et al., 2006; Uemura, et al., 2006; Gnecchi, et al., 2008; Chimenti, et al., 2010; Maxeiner, et al., 2010; Huang, et al., 2011). The latter is likely promoted by local factors present in the ischemic tissue, one of which may be adenosine.

Adenosine, an endogenous nucleoside molecule, is released from cells or generated in the extracellular space as a result of breakdown of adenine nucleotides in many pathological conditions including hypoxia, cell stress, and injury (Fredholm, 2007). Concentrations of extracellular adenosine were demonstrated to increase in ischemic hearts, where it becomes a part of pathological environment (Martin, et al., 1997; Willems, et al., 2006). Adenosine exerts its actions via cell surface receptors of the G
protein-coupled receptor family, namely $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Fredholm, et al., 2001). Adenosine was suggested to affect neovascularization in various tissues by regulating the release of cytokines and growth factors (Adair, 2005). In particular, we have previously demonstrated that adenosine can play the role of a local regulator of angiogenesis in ischemic muscle tissue (Ryzhov, et al., 2007).

In the current study, we focused on a potential role of adenosine receptors in mouse cardiac stromal cells expressing stem cell antigen (Sca)-1 but lacking the endothelial marker CD31, a cell population reportedly capable of promoting neovascularization when injected in the ischemic heart (Wang, et al., 2006; Tateishi, et al., 2007; Matsuura, et al., 2009). Sca-1 is a cell surface marker commonly used for enrichment of adult murine stem/progenitor cell populations obtained from a wide variety of tissues and organs (Holmes and Stanford, 2007). Isolation of Sca-1$^+$CD31$^-$ multipotent cardiac stromal cells has been reported by several laboratories and their therapeutic potential was demonstrated in experimental myocardial infarction models (Huang, et al., 2011; Matsuura, et al., 2004; Pfister, et al., 2005; Wang, et al., 2006; Mohri, et al., 2009; Liang, et al., 2010; Matsuura, et al., 2009). However, the role of adenosine receptors in these cells has not been investigated. Therefore, we sought to characterize the expression of adenosine receptors on Sca-1$^+$CD31$^-$ multipotent cardiac stromal cells and test the hypothesis that their stimulation promotes secretion of proangiogenic factors.
METHODS

**Reagents.** Hams F12, DMEM high glucose (HG), DMEM low glucose (LG), Iscove’s modified Dulbecco’s medium, horse serum, and insulin–transferrin–selenium supplement were purchased from Invitrogen Corporation (Carlsbad, CA). Porcine transforming growth factor β1 (TGF-β1), mouse interferon γ (IFNγ) and 8-[4-[4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB-603) (Borrmann, et al., 2009) were purchased from R&D Systems (Minneapolis, MN). Mouse basic fibroblast growth factor (bFGF) was obtained from ProSpec-Tany Technogene, Ltd. (East Brunswick, NJ). EBM-2 Basal Medium and EGM-2 SingleQuot Kit supplement/growth factors were purchased from Lonza Walkersville, Inc. (Walkersville, MD), and EGM Cell Growth Medium-2 was prepared according the manufacture’s instruction. 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine (SCH58261) (Zocchi, et al., 1996) was a gift from Drs C. Zocchi and E. Ongini (Schering Plough Research Institute, Milan, Italy). 5′-N-ethylcarboxamidoadenosine (NECA) (Fredholm, et al., 2001), dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine (IBMX), L-ascorbic acid, β-glycerophosphate, human insulin solution (10mg/ml), fetal bovine serum (FBS), non-essential amino acids and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). When used as a solvent, final DMSO concentrations in all assays did not exceed 0.1% and the same DMSO concentrations were used in vehicle controls.

**Animals.** All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Animal studies were reviewed and approved by the institutional
animal care and use committee of Vanderbilt University. A2B receptor knockout (A2BKO) mice were obtained from Deltagen (San Mateo, CA) and wild-type (WT) C57BL/6 mice were purchased from Harlan World Headquarters (Indianapolis, IN). Genotyping protocols for A2BKO have been previously described (Csoka, et al., 2007). All of the A2BKO mice used in these studies were back-crossed to the C57BL/6 genetic background for more than 10 generations. Adenosine deaminase (ADA)-deficient mice (Blackburn, et al., 1998) were maintained on ADA enzyme therapy as described previously (Blackburn, et al., 2000) until age of 6 weeks. Increase in adenosine levels in the hearts was induced by withdrawing mice from enzyme therapy for 15 days before experiments (Willems, et al., 2006). The H-2Kb-tsA58 transgenic mice on C57BL/6 genetic background (Immortomouse®) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA).

Myocardial infarction. Surgical procedures to produce myocardial infarction in mice were performed in the Cardiovascular Pathophysiology and Complications Core of the Vanderbilt University Mouse Metabolic Phenotyping Center as previously described (Alfaro, et al., 2008). Anesthetized mice placed on an isothermal pad were maintained on artificial ventilation. After a left thoracotomy, a 7-0 suture was placed through the myocardium into the anterolateral left ventricular wall (around the left anterior descending artery) and the artery was ligated. The chest was closed in layers and the animals were gradually weaned from the respirator. Control sham-operated animals underwent the same surgical procedures except for artery ligation.

Mouse cardiac endothelial (CD31+) and Sca-1+CD31- stromal cells. Isolation of cardiac stromal cell populations was performed according to a previously published...
protocol (Wang, et al., 2006). In brief, hearts from five 6-8wk-old mice were dissected to isolate ventricular tissue, which was then minced and incubated with 10 ml of Digestion Solution (10 mg/ml Collagenase II, 2.5 U/ml Dispase II, 1 µg/ml DNase I and 2.5 mM CaCl2) for 20 min at 37°C. Filtered myocyte-free single cell suspension in PBS containing 0.5% BSA and 2 mM EDTA (PBS/BSA/EDTA) was treated with mouse BD Fc Block (clone 2.4G, BD Biosciences, San Jose, CA), and immune cells were magnetically removed with CD45 microbeads (Miltenyi Biotec, Inc., Auburn, CA). After incubation with phycoerythrin (PE)-conjugated CD31 (clone 390, eBioscience, Inc., CA) and fluorescein isothiocyanate (FITC)-conjugated Sca-1 (clone E13-161.7, BD Biosciences) antibodies, CD31-positive cells were collected with anti-PE Microbeads (Miltenyi Biotec). Sca-1⁺CD31⁻ cells were magnetically isolated with anti-FITC microbeads (Miltenyi Biotec) from the flow-through CD31-negative cells.

Primary cardiac endothelial (CD31⁺CD45⁻) cells were cultured on 1% gelatin-coated tissue culture dishes in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 10 U/ml heparin, and 50 µg/ml endothelial mitogen (ECGS; BT-203, Biomedical Technologies Inc., MA) under humidified atmosphere of air/CO₂ (19:1) at 37°C.

Primary cardiac stromal (Sca-1⁺CD31⁻CD45⁻) cells were plated at a density of 10⁴ cell/cm² and cultured on 1% gelatin-coated tissue culture dishes in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine under humidified atmosphere of air/CO₂ (19:1) at 37°C.

WT and A₂BKO conditionally immortalized cardiac stromal (Sca-1⁺CD31⁻CD45⁻) cell lines were isolated as described above from H-2Kb-tsA58 transgenic mice crossed
with WT and A2BKO, mice respectively. Conditionally immortalized cells were propagated on 0.1% gelatin-coated tissue culture dishes in DMEM-(HG) supplemented with 10% FBS, 1X Antibiotic-Antimycotic solution (Sigma), 2 mM glutamine, and 10 ng/ml of IFNγ under humidified atmosphere of air/CO2 (19:1) at 33°C. Six days before experiments, cells were replated and cultured in the absence of IFNγ at 37°C.

**Human cardiac stromal cells.** Cell isolation method was adopted from a previously published protocol of clonogenic isolation of human cardiomyocyte progenitor cells (Smits, et al., 2009). All procedures for tissue procurement were performed in compliance with institutional guidelines for human research and an approved Institutional Review Board protocol at the Vanderbilt University. De-identified samples (0.5 – 1 cm³) of left ventricular tissue from 3 different patients undergoing heart transplantation were obtained through the Cardiology Core Laboratory for Translational and Clinical Research at Vanderbilt University and the Vanderbilt Heart Biorepository. Minced tissue was incubated in Digestion Solution for 20-25 min at 37°C. After passing through 70 μm cell strainer, the resulting myocyte-free single cell suspension was centrifuged at 500 x g, washed with Dulbecco’s PBS and resuspended in PBS/BSA/EDTA. Hematopoietic cells were removed by magnetic separation using human CD45 microbeads (Miltenyi Biotec). CD45-depleted cells were plated on 96-well plates at a density of 0.5 cell per well in M199/EGM-2 (3:1, v/v) supplemented with 10% FBS and 1X Antibiotic-Antimycotic solution. The wells were analyzed for growing colonies twice weekly. Rapidly growing clones (2-3 colonies per sample) were harvested, resuspended in a fresh growth medium, and plated on 0.1% gelatin-coated tissue culture dishes at a density of 5 x 10³ cells/cm². Cells were cultured under humidified atmosphere...
of air/CO₂ (19:1) at 37°C for 2-6 passages before experiments. One cell line per each tissue sample was arbitrarily chosen for further analysis.

**Induction of cell lineage commitment.** Differentiation of murine cardiac stromal cells toward adipogenic and osteogenic lineages was induced as previously described (Anjos-Afonso and Bonnet, 2008) with minor modifications. Briefly, Sca-1⁺CD31⁻ cells were plated onto 0.1% gelatin A coated 2-chamber slide (Lab-Tek® Chamber Slide™, Fisher Scientific, Pittsburgh, PA) at a density of 5 x 10³ cell/cm². Cells were grown in DMEM-LG medium supplemented with 10% FBS for 24-48 hrs to 80%-90% confluency. At this point, the growth medium was replaced with an appropriate differentiating medium and cells were cultured for an additional 2 weeks with differentiating medium changed every 3 days.

Cells directed toward an adipogenic lineage (DMEM-LG containing 2% FBS and supplemented with 1 μM dexamethasone, 5 μg/ml insulin, 50 μM indomethacin and 500 nM IBMX) were fixed for 30 min with 10% buffered formalin and stained with 0.21% Oil Red O (Sigma). Cells undergoing osteogenic (DMEM-LG, 2% FBS, 10 nM dexamethasone, 50 μM ascorbic acid and 10 mM β-glycerophosphate) differentiation were fixed for 15 min with 100% ethanol and stained with 0.2% Alizarin Red (Sigma).

Differentiation of human cardiac stromal cells toward cardyomyocyte lineage was conducted according to a previously described protocol (Smits, et al., 2009). To induce cardiomyogenic differentiation of murine cells, cardiac stromal cells were pre-treated with 5 μM 5′-azacytidine for 72 h in DMEM-LG medium supplemented with 10% FBS and cultured in DMEM-LG medium supplemented with 2% FBS, 1 ng/ml TGFβ1, 100
μM ascorbic acid, 0.2% DMSO and 10 ng/ml bFGF for 3 weeks with medium changed every 3 days.

**Flow cytometry.** All cells were analyzed either freshly isolated from ventricles or after treatment of monolayer cultures with Accutase-Enzyme Cell Detachment Medium (eBioscience). Cells (~5x10^5) were washed and resuspended in 100 μl of PBS/BSA/EDTA and 2 μl of either human or murine Fc block reagent (BD Biosciences). The cells were then incubated with relevant antibodies for 20 min at 4°C, washed once with 10 volumes of cold PBS/BSA/EDTA, and resuspended in a final volume of 500 μl. For intracellular vascular endothelial growth factor (VEGF) staining, myocyte-depleted single cell suspensions prepared from both right and left ventricles were incubated in DMEM containing 5% FBS and 3 μg/ml Brefeldin A (eBioscience) for 4 hours to block cytokine secretion. Cell surface antigens were stained with PE-conjugated anti-CD31, anti-Sca-1-PeCy7 (eBioscience) and anti-CD45-V450 (BD Biosciences) antibodies. After treatment with Cytofix/Cytoperm kit (BD Biosciences), the permeabilized cells were stained for VEGF using rabbit anti-VEGF-A (bs-1957R, Bioss-USA, Woburn, MA) and FITC-conjugated donkey anti-rabbit antibody (BioLegend, San Diego, CA). Rabbit IgG’s (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as an isotype control. Viable and non-viable cells were distinguished using LIVE/DEAD® fixable blue Stain kit (Life Technologies, Carlsbad, CA). Data acquisition was performed using LSRII flow cytometer (Becton Dickinson), and the data were analyzed with WinList 5.0 software. Antigen negativity was defined as having the same fluorescent intensity as the isotype control.
**Real-time reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed in triplicate for each sample with an ABI PRISM 7900HT Sequence Detection System (ABI Applied Biosystems, Foster City, CA). Primer pairs and FAM-labeled probes for human (A1, Hs00181231; A2A, Hs00169123; A2B, Hs00386491; A3, Hs00181223) and murine adenosine receptors (A1, Mm01308023; A2A, Mm00802075; A2B, Mm00839292; A3, Mm08802076), and β-actin (human β-actin, Hs99999903; murine β-actin, Mm00607939) were provided by Applied Biosystems. Each reaction was normalized against β-actin. Other primer sequences are listed in Table.

**Transfections.** The cDNA encoding the murine A2B adenosine receptor in the pcDNA3.1 expression vector was a generous gift from Dr. John A. Auchampach (Medical College of Wisconsin, Milwaukee, WI). A control plasmid pcDNA3.1 was purchased from Invitrogen Corporation. Conditionally immortalized Sca-1^+^CD31^-^ cells were transfected using Fugene 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN).

**Measurement of cAMP accumulation.** Cyclic AMP accumulation was measured by a cAMP-binding protein assay as previously described (Feoktistov, et al., 2002).

**Analysis of cytokine secretion.** Interleukin (IL)-6, CXCL1, IL-8 and VEGF concentrations in culture media were measured using ELISA kits (R&D Systems).

**Statistical Analysis.** Data were analyzed using the GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA) and presented as mean ± SEM. Comparisons between several treatment groups were performed using one-way ANOVA followed by
appropriate post-tests. Comparisons between two groups were performed using two-tailed unpaired $t$ tests. A $p$ value $< 0.05$ was considered significant.
RESULTS

Isolation and characterization of primary cardiac stromal Sca-1^+CD31^- and endothelial CD31^+ cells. To obtain cardiac stromal cell population expressing the marker of stem/progenitor cells Sca-1 but lacking the endothelial marker CD31, we followed a previously established protocol of sequential magnetic selection for cells expressing CD31 and Sca-1 on their surface (Wang, et al., 2006). As illustrated in Figure 1A (left panel), this procedure resulted in enrichment of Sca-1^+CD31^- cells up to approximately 80%. Cell population expressing the endothelial marker CD31^+, which included both Sca-1^- and Sca-1^+ subsets (22 and 78% of all CD31^+ cells, respectively), was enriched up to approximately 90% (Figure 1A, right panel) and used next as a reference cell population in the characterization of cardiac Sca-1^+CD31^- stromal cells.

The collected Sca-1^+CD31^- and CD31^+ cell populations were plated and expanded to confluency in serum-free or endothelial growth media, respectively, as described in Methods. Immunophenotyping of the expanded primary cells confirmed the strong expression of CD31 in CD31^+ cells and the absence of this marker in Sca-1^+CD31^- cells. Both Sca-1^+CD31^- and CD31^+ cell populations expressed Sca-1 and CD105 (endoglin) but lacked the expression of CD34, CD117 (c-kit), and CD45 (Figure 1B), displaying phenotypes previously reported for mesenchymal stem-like and endothelial cells, respectively (Dominici, et al., 2006; Tateishi, et al., 2007; Ieronimakis, et al., 2008; Lidington, et al., 2002). In addition, Sca-1^+CD31^- cells expressed medium to high levels of standard mesenchymal/stromal cell surface markers CD29 (integrin β-1), CD44 (H-CAM), CD73 (ecto-5'-nucleotidase), and CD90 (Thy-1) (data not shown).
Gene expression analysis revealed that Sca-1⁺CD31⁻ cells expressed higher levels of mRNA encoding stem/progenitor cell markers telomerase reverse transcriptase (TERT), polycomb group protein Bmi1, and ATP-binding cassette transporter Bcrp1 compared to CD31⁺ cells (Fig. 1C). These cardiac stromal cells, however, did not express the embryonic stem cell markers OCT4 or UTF1 (data not shown). Taken together, immunophenotype and gene expression pattern of Sca-1⁺CD31⁻ cells are in close agreement with previously reported characteristics of adult cardiac stem-like cells (Tateishi, et al., 2007).

Expression of adenosine receptors in primary cardiac stromal Sca-1⁺CD31⁻ and endothelial CD31⁺ cells. Real-time RT-PCR analysis revealed that Sca-1⁺CD31⁻ cells preferentially express mRNA encoding A2B receptors (Figure 1D). Much lower levels of A2A receptor mRNA were also detected, whereas transcripts for A₁ and A₃ receptors were below detection levels. In contrast, CD31⁺ cells exhibited much lower expression of A2B receptor mRNA levels, although the expression of A2A receptors was similar to that in Sca-1⁺CD31⁻ cells. Like in Sca-1⁺CD31⁻, no A₁ and A₃ receptor transcripts were detected in CD31⁺ cells.

A2A and A2B receptors are known to stimulate adenylate cyclase via coupling to Gₛ proteins (Fredholm, et al., 2001). Therefore, we measured cAMP accumulation as a way to determine if expression of mRNA translates into functional presence of adenosine receptors in Sca-1⁺CD31⁻ and CD31⁺ cardiac cell populations. As seen in Figure 1E, the non-selective adenosine receptor agonist NECA (10 μM) stimulated cAMP accumulation by 7.9 ± 0.7-fold in Sca-1⁺CD31⁻ and to a lesser extent, by 2.7 ± 0.2-fold, in CD31⁺ cells.
Adenosine receptor-dependent regulation of VEGF, CXCL1 and IL-6 secretion from primary cardiac stromal Sca-1\(^+\)CD31\(^-\) and endothelial CD31\(^+\) cells. To determine if adenosine receptors play a role in paracrine functions of cardiac stromal cells, we measured VEGF, CXCL1 and IL-6 release by Sca-1\(^+\)CD31\(^-\) and CD31\(^+\) cells incubated for 6 hours in the absence or presence of 10 \(\mu M\) NECA. In the absence of NECA, cardiac Sca-1\(^+\)CD31\(^-\) stromal cells produced 18-fold higher levels of VEGF compared with cardiac cells expressing the endothelial marker CD31 (Figure 1F, left panel). VEGF secretion from Sca-1\(^+\)CD31\(^-\) cells was augmented more than 3-fold by stimulation of adenosine receptors with NECA. In contrast, NECA had no significant effect on VEGF release from endothelial cells.

Both cardiac Sca-1\(^+\)CD31\(^-\) stromal and endothelial cells tonically release CXCL1; NECA further augmented CXCL1 secretion to similar levels (Figure 1F, middle panel). In the absence of NECA, cardiac Sca-1\(^+\)CD31\(^-\) stromal cells produced 5-fold higher levels of IL-6 compared with cardiac endothelial cells (Figure 1F, right panel). IL-6 secretion from both cell types was augmented 1.7-1.8-fold by stimulation of adenosine receptors with NECA.

Effect of myocardial infarction on populations of cardiac stromal and endothelial cells in vivo. Because cardiac Sca-1\(^+\)CD31\(^-\) stromal cells produced considerably higher levels of the angiogenic factor VEGF compared to CD31\(^+\) endothelial cells, we thought it was important to evaluate their relative numbers in the heart after myocardial infarction, a pathological condition that provides a potent stimulus for neovascularization. One week after permanent ligation of the left coronary artery, both left and right ventricles were dissected from infarcted and sham-operated hearts to prepare myocyte-depleted single
cell suspensions. Flow cytometry analysis of CD45-negative cardiac cells showed that the proportion of Sca-1^+CD31^- stromal cells was increased in the hearts underwent myocardial infarction one week earlier compared to control sham-operated hearts (Figure 2A). Based on absolute cell counts obtained by combining cell concentration determinations in each single cell suspension with corresponding flow cytometric population data, we conclude that Sca-1^+CD31^- stromal cell numbers are significantly increased whereas Sca-1^+CD31^+ endothelial cell numbers are significantly decreased in ventricles one week after coronary artery ligation (Figure 2B). A considerable increase in stromal to endothelial cell ratio indicates their relative functional importance at this stage after myocardial infarction.

**Analysis of VEGF production by cardiac Sca-1^+CD31^- stromal and Sca-1^+CD31^+ endothelial cells induced by adenosine in vivo.** Withdrawal from ADA enzyme replacement therapy for two weeks has been reported to increase adenosine levels in hearts of ADA-deficient mice (Willems, et al., 2006). We used this model to evaluate in vivo the effects of adenosine on VEGF production by cardiac stromal and endothelial cells. Flow cytometry analysis of intracellular VEGF staining confirmed a greater VEGF production in cardiac Sca-1^+CD31^- stromal cells compared to Sca-1^+CD31^+ endothelial cells. Furthermore, VEGF production was significantly increased in cardiac Sca-1^+CD31^- stromal cells obtained from ADA-deficient mice withdrawn from ADA therapy (ADA-) compared to those obtained from control mice, which continued to receive ADA replacement therapy (ADA+). In contrast, no significant difference was found in VEGF production by cardiac Sca-1^+CD31^+ endothelial cells obtained from ADA- and ADA+ experimental groups (Figure 3).
Generation of conditionally immortalized cardiac Sca1⁺CD31⁻ cells. Primary cardiac Sca-1⁺CD31⁻ cells could be maintained in culture only for two or three passages before reaching senescence. To obtain conditionally immortalized Sca-1⁺CD31⁻ cells, we isolated them from hearts of Immortomouse® (Charles River) containing a gene encoding the thermolabile SV40 T antigen (Jat, et al., 1991). The inducible tsA58 TAg gene allows these cells to be rapidly expanded under permissive conditions (at 33°C in the presence of IFN-γ), so as to obtain sufficient Sca-1⁺CD31⁻ cells for analysis. Conditionally immortalized cells were grown for 4-8 passages. Experiments were then performed with cells switched to their untransformed state by culture at 37°C in the absence of IFN-γ (i.e. non-permissive conditions).

Figure 4A shows phase contrast (upper panels) and immunofluorescence (lower panels) micrographs of Sca-1⁺CD31⁻ cells derived from Immortomouse® hearts. As seen in the lower left panel, Sca-1⁺CD31⁻ cells cultured at 33°C with IFN-γ demonstrated abundant nuclear staining with anti-SV40 large T antigen MAb PAb101 (Pharmingen, San Diego, CA). However, after 48 h at 37°C in the absence of IFN-γ, tsA58 TAg expression was undetectable (faint non-specific staining in the lower right panel). Flow cytometry analysis confirmed that the expression of cell surface markers on these cells was similar to that found on primary cells (Fig 4B).

To verify if the immortalized cells preserved the reported multipotent capacities of primary cardiac stromal cells (Matsuura, et al., 2004; Tateishi, et al., 2007), we determined their adipogenic, osteogenic, and cardiomyogenic potential. Alizarin red and Oil Red O positive staining indicative of induction of osteogenic and adipogenic differentiation, respectively, was observed in immortalized cardiac Sca-1⁺CD31⁻ cells.
cultured for 2 weeks in corresponding differentiating media but not in cells cultured in a standard control medium at 37°C (Figure 4C, and see Methods). Upregulation of several cardiac-specific genes, including transcription factors Nkx2.5, Mef2C, cardiac TnT, α-actin and β-myosin heavy chains, was also evident in immortalized cardiac Sca-1⁺CD31⁻ cells cultured in cardiomyogenic differentiation medium for 3 weeks compared to cells cultured in a standard control medium for the same period (Figure 4D, and see Methods). These changes in the phenotype of immortalized cardiac Sca-1⁺CD31⁻ cells under corresponding differentiating conditions are in close agreement with changes previously reported in primary cardiac stromal cells (Matsuura, et al., 2004; Tateishi, et al., 2007).

Role of A₂B receptors in adenosine-dependent secretion of paracrine factors from cardiac Sca-1⁺CD31⁻ cells. Like primary cells, conditionally immortalized WT cardiac Sca-1⁺CD31⁻ cells preferentially express mRNA encoding A₂B receptors. As expected, conditionally immortalized cells derived from A₂BKO mice did not express mRNA encoding A₂B receptors. Low levels of A₂A receptor mRNA were detected in both WT and A₂BKO Sca-1⁺CD31⁻ cells, whereas transcripts for A₁ and A₃ receptors were below detection levels (Figure 5A).

Stimulation of adenosine receptors with increasing concentrations of NECA induced accumulation of cAMP in WT Sca-1⁺CD31⁻ cells with an estimated EC₅₀ value of 170 nM but had no significant effect in cells lacking A₂B receptors (Figure 5B). NECA also stimulated VEGF production in a concentration-dependent manner in WT cardiac Sca-1⁺CD31⁻ cells with an estimated EC₅₀ value of 33 nM but had no effect in A₂BKO cells (Figure 5C). Stimulation of adenosine receptors with 10 μM NECA significantly increased CXCL1 release from WT Sca-1⁺CD31⁻ cells but had no effect on CXCL1.
secretion in A2BKO cells (Figure 5D). Similarly, stimulation of adenosine receptors with NECA significantly increased IL-6 release from WT cardiac Sca-1⁺CD31⁻ cells but had no effect on IL-6 secretion in A2BKO cells (Figure 5E). NECA effects were rescued by transient expression of A2B receptors in A2BKO cells; transfection with a plasmid encoding mouse A2B receptors but not with an empty vector enabled these cells to increase IL-6 release in response to NECA (Figure 5F). Taken together, our results suggest that A2B receptors mediate the adenosine-induced secretion of VEGF, CXCL1 and IL-6 from cardiac Sca-1⁺CD31⁻ cells.

**Adenosine-dependent regulation of human cardiac stromal cells.** Sca-1 is not expressed in humans (Holmes and Stanford, 2007) but multipotent stromal cells similar to their murine counterparts have been described in the human heart (Messina, et al., 2004; Smits, et al., 2009). To determine if adenosine receptors regulate paracrine functions of these cells, we isolated a highly proliferative stromal cell population from human cardiac tissue using a previously described single-cell clonogenic technique (Smits, et al., 2009). Figure 6A shows that cells obtained by this technique continued to grow rapidly in culture expanding from 5%-10% confluency on day 0 to 60%-70% confluency on day 3. Like mouse cardiac Sca-1⁺CD31⁻ cells, the human stromal cells expressed high levels of CD105 on their surface and were negative for the common immune cell surface marker CD45 as well as the hematopoietic progenitor markers CD34 and CD117. These cells also expressed intermediate levels of CD90 and very low levels of CD31 (Figure 6B). Incubation of human cardiac stromal cells in a cardiacmyogenic medium for 2 weeks increased the expression of cardiac-specific genes GATA-4, Nkx2.5, Mef2C, cardiac
troponin T, α-actin and β-myosin heavy chains. Inclusion of 10 μM NECA in a cardiomyogenic medium had no considerable effect on their expression (Figure 6C).

Real-time RT-PCR analysis of human cardiac stromal cells revealed preferential expression of mRNA encoding A2B receptors, with lower expression of A2A receptors and no detectable levels of A1 and A3 receptor transcripts (Figure 6D). Stimulation of adenosine receptors on human cardiac stromal cells with 10 μM NECA for 6 hours significantly increased release of IL-6, IL-8 and VEGF. Adenosine-dependent release of these factors was significantly inhibited by the selective A2B receptor antagonist PSB-603, but not the selective A2A receptor antagonist SCH52861 (Figure 6E).
DISCUSSION

Extracellular accumulation of adenosine in response to myocardial ischemia and tissue damage is an important event in the control of many aspects of tissue repair, including revascularization. This study has demonstrated that A<sub>2B</sub> adenosine receptors can regulate paracrine functions of cardiac mesenchymal stem-like cells involved in regulation of angiogenesis. Our study revealed that cardiac Sca-1<sup>+</sup>CD31<sup>-</sup> stromal cells predominantly express mRNA encoding the A<sub>2B</sub> adenosine receptor subtype and considerably lower levels of A<sub>2A</sub> adenosine receptor transcripts. Importantly, stimulation of adenosine receptors promoted release of the major proangiogenic factor VEGF from Sca-1<sup>+</sup>CD31<sup>-</sup> stromal cells. Stimulation of adenosine receptors on Sca-1<sup>+</sup>CD31<sup>-</sup> stromal cells also increased the release of CXCL1 and IL-6, factors known to promote angiogenesis (Strieter, et al., 1995; Hernandez-Rodriguez, et al., 2003). Using conditionally immortalized mouse cardiac Sca-1<sup>+</sup>CD31<sup>-</sup> stromal cell lines, we demonstrated that stimulation of adenosine receptors increased CXCL1, IL-6 and VEGF secretion only in WT but not A<sub>2B</sub>KO cells. Conversely, the loss of adenosine-dependent IL-6 secretion in A<sub>2B</sub>KO cells was reversed by transient expression of recombinant A<sub>2B</sub> receptors. Therefore, we concluded that A<sub>2B</sub> receptors are essential for adenosine-dependent upregulation of paracrine functions of Sca-1<sup>+</sup>CD31<sup>-</sup> stromal cells which may serve as an important source of proangiogenic stimuli in the heart.

Initial evidence that resident mesenchymal stem-like cells can play an important role in repair of injured heart came from reports that injection of mouse cardiac Sca-1<sup>+</sup>CD31<sup>-</sup> stromal cells but not CD31<sup>+</sup> endothelial cells improved cardiac function and promoted angiogenesis after experimental myocardial infarction (Wang, et al., 2006). It
has been suggested that Sca-1⁺CD31⁻ cells but not CD31⁺ cells are capable to transdifferentiate into cardiomyocytes (Pfister, et al., 2005). However, the paracrine functions of these cells may be also important in their therapeutic effects, as evidenced by the observation that conditioned media collected from cardiac Sca-1⁺CD31⁻ stromal cells improved cardiac function in a mouse myocardial ischemia/reperfusion model (Huang, et al., 2011). The present study shows that cardiac Sca-1⁺CD31⁻ stromal cells not only express higher levels of stem cell/progenitor markers but also release higher levels of VEGF compared to cardiac CD31⁺ endothelial cells. Contribution of Sca-1⁺CD31⁻ stromal cells to VEGF production in the heart can be even greater after myocardial infarction. It has been previously shown that population of Sca-1⁺CD31⁻ cells is significantly increased by day 7 after myocardial infarction (Wang, et al., 2006). Our data show that an increase in numbers of Sca-1⁺CD31⁻ stromal cells occurs simultaneously with a decrease in Sca-1⁺CD31⁺ endothelial cell numbers. These results can be explained by potential massive death of endothelial cells with concomitant proliferation of stromal cell, and/or by endothelial-to-mesenchymal transition induced by myocardial infarction as demonstrated in our recent study (Aisagbonhi, et al., 2011). Myocardial infarction would also lead to accumulation of interstitial adenosine, which in turn would further increase VEGF production by cardiac Sca-1⁺CD31⁻ stromal cells. We explored the in vivo relevance of our findings in ADA-deficient mice. Once withdrawn from enzyme replacement therapy, adenosine levels begin to rise in all organs of these mice including the heart (Blackburn et al., 2000; Willems et al., 2006). Of relevance to the present study, the levels of adenosine in ADA-deficient hearts two weeks after withdrawal from ADA replacement therapy were shown to be comparable to those.
produced by ischemic injury in normal hearts (Willems et al., 2006). Although adenosine is only a part of complex milieu produced by myocardial infarction, the increase in adenosine levels in these mice was sufficient to induce an increase in VEGF production in cardiac Sca-1⁺CD31⁻ stromal cells but not in Sca-1⁺CD31⁺ endothelial cells.

In vitro, cardiac endothelial cells failed to significantly increase VEGF production in response to stimulation with NECA despite the expression of functional A₂ adenosine receptors. We have previously reported that stimulation of adenosine receptors upregulated VEGF production in retinal and skin microvascular endothelial cells but had no effect in human umbilical vein endothelial cells (Grant, et al., 1999; Grant, et al., 2001; Feoktistov, et al., 2002). Our results, therefore, are consistent with heterogeneity of adenosine-dependent regulation of VEGF production in different types of endothelial cells. It is remarkable that VEGF release from cardiac Sca-1⁺CD31⁻ stromal cells was considerably higher than from cardiac CD31⁺ endothelial cells. These results suggest that adenosine can stimulate secretion of paracrine factors from Sca-1⁺CD31⁻ stromal cells, whereas release of autocrine VEGF from cardiac endothelial cells remains low. Thus, adenosine can contribute to a gradient of VEGF in the damaged heart toward which new vessels would grow.

VEGF is considered to be a key regulator of angiogenesis (Ferrara, et al., 2003). We have demonstrated previously that specific inhibition of VEGF with a neutralizing antibody completely blocked proangiogenic effects of conditioned media collected from adenosine-stimulated mast cells, despite the presence of other proangiogenic factors (Feoktistov, et al., 2003). In this study, we found that stimulation of adenosine receptors in both cardiac stromal and endothelial cells upregulated secretion of CXCL1 and IL-6,
albeit to a different extent. It is possible, therefore, that endothelial cells can also contribute to adenosine-dependent stimulation of angiogenesis by releasing these cytokines with reported proangiogenic activity, which may facilitate the actions of VEGF released primarily from stromal cells.

Our study also revealed that the human heart harbors a stromal cell population similar to the mouse cardiac Sca-1^+CD31^- stromal cells in respect of adenosine-dependent regulation of their paracrine functions. We found that human mesenchymal stem-like cells predominantly express A_{2B} receptors and respond to stimulation with NECA by increased release of IL-6, IL-8 and VEGF. These effects were inhibited by the selective A_{2B} receptor antagonist PSB-603 but not the selective A_{2A} antagonist SCH52861. Thus, like in mouse cardiac Sca-1^+CD31^- stromal cells, stimulation of A_{2B} adenosine receptors on human cardiac mesenchymal stem-like cells increases their secretion of proangiogenic factors.

In contrast to stimulation of IL-6, IL-8 and VEGF secretion, we found no evidence of A_{2B} receptor-dependent regulation of cardiomyogenic differentiation of cardiac stromal cells in vitro. Inclusion of NECA in cardiomyogenic medium did not affect the expression of cardiac-specific genes in differentiating cells. However, considering a highly artificial nature of cardiomyogenic differentiation in vitro, caution should be taken in extrapolating these results to the actual situation in vivo.

In this study, we did not address the role of signaling mechanisms downstream from the A_{2B} receptor in regulation of proangiogenic factors in cardiac stromal cells. Although we have demonstrated NECA-induced accumulation of cAMP as a way to determine if A_{2} receptors remain functional in the isolated cardiac cells, our data do not
necessarily imply that stimulation of all proangiogenic factors is cAMP-dependent. A2B receptors have been linked to activation of not only Gs but also Gq proteins regulating cAMP-independent pathways (Feoktistov and Biaggioni, 1995; Linden, et al., 1999; Ryzhov, et al., 2009). Furthermore, our previous studies in other cells have demonstrated that intracellular signaling pathways involved in the A2B receptor-dependent stimulation are complex and can be different for specific proangiogenic factors (Feoktistov, et al., 1999; Ryzhov, et al., 2006). For example, we have reported previously that A2B receptor-mediated stimulation of VEGF production in mast cells was only in part cAMP-dependent (Ryzhov, et al., 2008b), whereas stimulation of IL-8 production was not affected at all by inhibition or activation of cAMP-dependent pathways (Feoktistov and Biaggioni, 1995; Ryzhov, et al., 2006). It remains to be elucidated which intracellular signaling pathways triggered by activation of A2B receptors would regulate production and release of specific proangiogenic factors from cardiac stromal cells.

Taken together, our results contribute to the growing evidence that A2B receptors play an important role in neovascularization. We have shown previously that A2B receptors upregulate proangiogenic factors in retinal and skin endothelial cells (Grant, et al., 1999; Grant, et al., 2001; Feoktistov, et al., 2002), certain types of cancer cells (Zeng, et al., 2003; Ryzhov, et al., 2008a), mast cells (Feoktistov and Biaggioni, 1995; Feoktistov, et al., 2003; Ryzhov, et al., 2008b), and tumor-infiltrating hematopoietic cells (Ryzhov, et al., 2008a). Now we demonstrate that mouse cardiac Sca-1^+CD31^- stromal cells express functional A2B receptors, which are linked to upregulation of proangiogenic factors and that similar A2B receptor-dependent regulation exists in mesenchymal stem-like cells derived from the human heart. Thus, our study identified A2B adenosine
receptors on cardiac stromal cells as potential targets for upregulation of proangiogenic factors in the heart. It remains to be determined if stimulation and/or upregulation of A$_{2B}$ adenosine receptors on mesenchymal stem-like cells could boost their beneficial effects in cell-based approaches to treatment of cardiovascular disease.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Ryzhov, Novitskiy and Feoktistov

Conducted experiments: Ryzhov, Goldstein and Novitskiy

Contributed new reagents or analytical tools: Blackburn

Performed data analysis: Ryzhov and Feoktistov

Wrote or contributed to the writing of the manuscript: Ryzhov, Novitskiy, Biaggioni and Feoktistov
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(2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. 

*Circ Res* **95**:911-921.


**FOOTNOTES:**

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FIGURE LEGENDS

Figure 1. Isolation and characterization of primary cardiac Sca-1⁺CD31⁻ and CD31⁺ cells.

A, Representative cytofluorographic dot plots of CD31 and Sca-1 cell-surface expression on CD45⁻ cells isolated by magnetic sorting for CD31⁺ marker (right panel) and for Sca-1⁺ marker from the CD31-depleted cells (left panel). Please see Methods for details.

B. Flow cytometry histograms of cell surface markers on Sca-1⁺CD31⁻ and CD31⁺ cells. Shaded areas represent the fluorescence of cells treated with corresponding isotype-matching antibody controls.

C, Real-time RT-PCR analysis of mRNA encoding stem/progenitor cell markers telomerase reverse transcriptase (TERT), polycomb group protein Bmi1, and ATP-binding cassette transporter Bcrp1 in Sca-1⁺CD31⁻ and CD31⁺ cells. Values are means±SEM of 3 experiments.

D, Real-time RT-PCR analysis of mRNA encoding adenosine receptors in Sca-1⁺CD31⁻ and CD31⁺ cells. Values are averages of 2 experiments.

E, Cyclic AMP accumulation in Sca-1⁺CD31⁻ and CD31⁺ cells in the absence (Basal) or presence of 10 μM NECA. Values are means±SEM of 3 experiments.

F, VEGF, CXCL-1, and IL-6 release from Sca-1⁺CD31⁻ and CD31⁺ cells in the absence (Basal) or presence of 10 μM NECA. Values are means±SEM of 3 experiments. Asterisks indicate statistical difference (*p<0.05, **p<0.01, ***p<0.001, unpaired two-tail t-test) and ns indicates non-significant difference compared to basal levels.

Figure 2. Myocardial infarction induces an increase in cardiac Sca-1⁺CD31⁻ stromal and a decrease in Sca-1⁺CD31⁺ endothelial cell populations.
A, Representative cytofluorographic dot plots of CD31 and Sca-1 cell-surface staining of CD45- cells from mouse hearts 7 days after experimental myocardial infarction (post-MI) or sham (Control) surgery.

B, Total numbers of Sca-1+CD31- stromal cells and endothelial CD31+ cells calculated from data shown in panel A and total numbers of CD45-negative cells in each mouse heart. Values are means±SEM of 4 experiments. Asterisks indicate statistical differences (*p<0.05, unpaired two-tail t-test) compared to control.

**Figure 3. Effect of increased adenosine levels in ADA-deficient mice on VEGF production in cardiac Sca-1+CD31- stromal and Sca-1+CD31+ endothelial cells.**

A, Representative flow cytometry analysis of intracellular VEGF staining in cardiac Sca-1+CD31- and Sca-1+CD31+ cell populations from ADA-deficient mice maintained on ADA enzyme therapy (ADA+) or withdrawn from therapy for 15 days to induce an increase in myocardial adenosine levels (ADA-).

B, Graphic representation of data from flow cytometry analysis of intracellular VEGF staining of cardiac Sca-1+CD31- and Sca-1+CD31+ cell populations from ADA+ or ADA- mice. Values are means±SEM of 4 experiments. Asterisks indicate statistical differences (***p<0.01, unpaired two-tail t-test) and ns indicates non-significant difference compared to ADA+.

**Figure 4. Characterization of conditionally immortalized cardiac Sca-1+CD31- cells.**

A, Phase contrast (upper panels) and tsA58 TAg immunofluorescent (lower panels) micrographs of the expanded immortalized cardiac Sca-1+CD31- cells cultured at 33°C in the presence of IFN-γ (left panels) or at 37°C in normal growth medium (right panels) for 48 h. The bar represents 100 μm.
B. Flow cytometry histograms of cell surface markers on the expanded immortalized cardiac Sca-1^+CD31^− cells cultured at 37°C in normal growth medium for 48 h. Shaded areas represent the fluorescence of cells treated with corresponding isotype-matching antibody controls.

C. Micrographs of immortalized cardiac Sca-1^+CD31^− cells cultured at 37°C in normal growth medium (Control), or in respective adipogenic or osteogenic differentiating media (Diff) as described in the Methods section. Adipogenesis and osteogenesis were indicated by Oil Red and Alizarin Red, respectively. Scale bar = 100 μm.

D. Real-time RT-PCR analysis of cardiac-specific gene expression in immortalized cardiac Sca-1^+CD31^− cells cultured at 37°C in normal growth medium (Con), or in differentiating media (Diff) for 1 or 3 weeks (w) as described in the Methods section. Values are averages of 2 experiments.

**Figure 5. Characterization of adenosine receptor subtypes in cardiac Sca-1^+CD31^− cells.**

A. Real-time RT-PCR analysis of mRNA encoding adenosine receptors in WT and A2BKO immortalized cardiac Sca-1^+CD31^− cells. Values are averages of 2 experiments.

B. Effect of increasing NECA concentrations on cAMP accumulation in WT and A2BKO immortalized cardiac Sca-1^+CD31^− cells. Values are expressed as mean±SEM, n=3. Asterisks indicate significant difference (**p<0.01, one-way ANOVA with Dunnett's post-test) compared to basal values.

C. Effect of increasing NECA concentrations on VEGF release from WT and A2BKO immortalized cardiac Sca-1^+CD31^− cells. Values are expressed as mean±SEM, n=3.
Asterisks indicate significant difference (\(**p<0.01\), one-way ANOVA with Dunnett's post-test) compared to basal values.

D, CXCL1 release from WT and A\(_{2B}\)KO immortalized cardiac Sca-1\(^+\)CD31\(^-\) cells in the absence (Basal) or presence of 10 \(\mu\)M NECA. Values are means±SEM of 3 experiments. Asterisks indicate statistical difference (\(***p<0.001\), unpaired two-tail t-test) and ns indicates non-significant difference compared to basal levels.

E, IL-6 release from WT and A\(_{2B}\)KO immortalized cardiac Sca-1\(^+\)CD31\(^-\) cells in the absence (Basal) or presence of 10 \(\mu\)M NECA. Values are means±SEM of 3 experiments. Asterisks indicate statistical difference (\(***p<0.001\), unpaired two-tail t-test) and ns indicates non-significant difference compared to basal levels.

F, IL-6 release from A\(_{2B}\)KO immortalized cardiac Sca-1\(^+\)CD31\(^-\) cells transfected either with plasmid encoding mouse A\(_{2B}\) adenosine receptor (+A\(_{2B}\)AR) or with an empty vector (Mock) measured in the absence (Basal) or presence of 10 \(\mu\)M NECA. Values are means±SEM of 3 experiments. Asterisk indicates statistical difference (\(*p<0.05\), unpaired two-tail t-test) and ns indicates non-significant difference compared to basal levels.

Figure 6. Characterization of human primary cardiac stromal cells.

A, Phase contrast micrographs of human cardiac stromal cells immediately after replating a single cell-derived clone (d0, upper panel) and 3 days later (d3, lower panel). Scale bar = 100 \(\mu\)m.

B, Flow cytometry histograms of cell surface markers on human cardiac stromal cells. Shaded areas represent the fluorescence of cells treated with corresponding isotype-matching antibody controls.
C, Real-time RT-PCR analysis of cardiac-specific gene expression in human cardiac stromal cells before (C) and after culturing in differentiating media for 1 or 2 weeks (w) in the absence (Vehicle) or presence of 10 μM NECA. Values are averages of 2 experiments.

D, Real-time RT-PCR analysis of mRNA encoding adenosine receptors in human cardiac stromal cells. Values are averages of 2 experiments.

E, IL-6, IL-8 and VEGF release from human cardiac stromal cells in the absence (Basal) or presence of 10 μM NECA, or in the presence of NECA and 100 nM SCH58261 or 100 nM PSB-603. Values are means±SEM of 3 experiments. Asterisks indicate statistical difference (**:p<0.01, and ***:p<0.001) compared to basal levels, daggers indicate statistical difference (†:p<0.05, and †††:p<0.001) and ns indicates non-significant difference compared to NECA-stimulated levels by one-way ANOVA with Bonferroni post-test.
### Table. Primers for RT-PCR used in this study.

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| **Human** |
| GATA-4 | TTCAAACCAGAAAGCGGAAG | CTGTGCCCCGTAAGTGATGA |
| Nkx2.5 | GCCTTCTATCCAGGTCTAC  | GCTCGCCTCTTGCTCTTCTCAG |
| MEF2C | AGATACACCAACACACACACACACGCGC   | ATCTTTTACAGAGTCTGAGTCTG |
| cTnT  | GTGGGAAGGACGAGGACTGAG | ATAGATGCTCTGCCACAGC |
| α-actin | TCCGTGCTCGCATTTTATTC | AACCCACTGCTCTAGCCACAG |
| β-MHC | GGAGGAGGACGAGGAAAAACC | CTTGCGGAACTTGAGGACAGG |
| β-actin | CCCCCAGGGACCGGAGG | GGCTGGGCTGGTGAAGG |

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Figure 1
Figure 2

(A) Flow cytometry analysis showing CD31 and Sca-1 expression in control and post-MI groups. The percentage of cells positive for CD31 and Sca-1 in each group is indicated.

(B) Bar graph showing the number of Sca-1^+CD31^- and Sca-1^+CD31^+ cells in control and post-MI groups. The bars indicate the mean ± SEM, and the asterisk denotes a statistically significant difference.
Figure 3

A

ADA+

ADA-

CD31

Sca-1

VEGF

B

VEGF, ΔMFI

Sca-1^CD31^  Sca-1^CD31^+

ADA+  ADA-

**  NS

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4
Figure 5