Role of A_{2B} Adenosine Receptors in Regulation of Paracrine Functions of Stem Cell Antigen 1-Positive Cardiac Stromal Cells

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ABSTRACT

The existence of multipotent cardiac stromal cells expressing stem cell antigen (Sca)-1 has been reported, and their proangiogenic properties have been demonstrated in myocardial infarction models. In this study, we tested the hypothesis that stimulation of adenosine receptors on cardiac Sca-1⁺ cells up-regulates their secretion of proangiogenic factors. We found that Sca-1 is expressed in subsets of mouse cardiac stromal CD31[−] and endothelial CD31⁺ cells. The population of Sca-1⁺CD31⁺ endothelial cells was significantly reduced, whereas the population of Sca-1⁺CD31[−] stromal cells was increased 1 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 vascular endothelial growth factor (VEGF) production in cardiac Sca-1⁺CD31[−] stromal cells but not in Sca-1⁺CD31⁺ endothelial cells. We found

that mouse cardiac Sca-1⁺CD31[−] stromal cells predominantly express mRNA encoding A_{2B} adenosine receptors. Stimulation of adenosine receptors significantly increased interleukin (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 A_{2B} receptor knockout mouse hearts, we demonstrated that A_{2B} receptors are essential for adenosine-dependent up-regulation 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 A_{2B} receptor stimulation. Thus, our study identified A_{2B} adenosine receptors on cardiac stromal cells as potential targets for up-regulation 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

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into different cell lineages (Beltrami et al., 2003; Matsuura et al., 2004, 2009; Messina et al., 2004; Pfister et al., 2005; Wang et al., 2006; Tateishi et al., 2007; Liang et al., 2010; 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 improved neovascularization and attenuated the decline of cardiac function in animal models of myocardial infarction (Messina et al., 2004; Wang et al., 2006; Tateishi et al., 2007; Martin et al., 2008; Matsuura et al., 2009; Huang et al., 2011). However, the early assumption that these cells can replace damaged car-

ABBREVIATIONS: Sca-1, stem cell antigen-1; DMEM, Dulbecco's modified Eagle's medium; HG, high glucose; LG, low glucose; IFN γ , interferon γ ; PSB-603, 8-[4-[4-(4-chlorophenyl)piperazine-1-sulfonyl]phenyl]-1-propylxanthine; SCH58261, 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine; NECA, 5'-N-ethylcarboxamidoadenosine; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; A_{2B}KO, A_{2B} adenosine receptor knockout; WT, wild type; ADA, adenosine deaminase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcription-polymerase chain reaction.

diomyocytes has recently given way to the realization that they also, 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; Gneccchi et al., 2008; Chimenti et al., 2010; Maxeiner et al., 2010; Huang et al., 2011). The latter is probably 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 the 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 (Matsuura et al., 2004, 2009; Pfister et al., 2005; Wang et al., 2006; Mohri et al., 2009; Liang et al., 2010; Huang et al., 2011). 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.

Materials and Methods

Reagents. Ham's F-12, 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 (Carlsbad, CA). Porcine transforming growth factor β 1, mouse interferon γ (IFN- γ), and 8-[4-[4-(4-chlorophenyl)piperazine-1-sulfonyl]phenyl]-1-propylxanthine (PSB-603) (Borrmann et al., 2009) were purchased from R&D Systems (Minneapolis, MN). Mouse basic fibroblast growth factor was obtained from ProSpec-Tany Technology, 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 to the manufacturer's instructions. 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-Ethylcarboxamido-adenosine (NECA) (Fredholm

et al., 2001), dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine, L-ascorbic acid, β -glycerophosphate, human insulin solution (10 mg/ml), fetal bovine serum (FBS), nonessential amino acids, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). When DMSO was used as a solvent, the final 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* (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health. Animal studies were reviewed and approved by the institutional animal care and use committee of Vanderbilt University. A_{2B} receptor knockout (A_{2B} KO) 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 A_{2B} KO have been described previously (Csoka et al., 2007). All of the A_{2B} KO 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 the age of 6 weeks. An 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-2K^b-tsA58 transgenic mice on a 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 described previously (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 coronary 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 protocol published previously (Wang et al., 2006). In brief, hearts from five 6- to 8-week-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 μ g/ml DNase I, and 2.5 mM CaCl₂) for 20 min at 37°C. A 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, San Diego, 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 μ g/ml endothelial mitogen (Biomedical Technologies, Cambridge, MA) under a 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 a humidified atmosphere of air/CO₂ (19:1) at 37°C.

WT and A_{2B}KO conditionally immortalized cardiac stromal (Sca-1⁺CD31⁻CD45⁻) cell lines were isolated as described above from H-2K^b-tsA58 transgenic mice crossed with WT and A_{2B}KO mice, respectively. Conditionally immortalized cells were propagated on 0.1% gelatin-coated tissue culture dishes in DMEM-HG supplemented with 10% FBS, 1× antibiotic-antimycotic solution (Sigma-Aldrich), 2 mM glutamine, and 10 ng/ml IFN-γ under a humidified atmosphere of air/CO₂ (19:1) at 33°C. Six days before experiments, cells were replated and cultured in the absence of IFN-γ at 37°C.

Human Cardiac Stromal Cells. The 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. Deidentified samples (0.5–1 cm³) of left ventricular tissue from three 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 to 25 min at 37°C. After passing through a 70-μm cell strainer, the resulting myocyte-free single-cell suspension was centrifuged at 500g, washed with Dulbecco's PBS, and resuspended in PBS/BSA/EDTA. Hematopoietic cells were removed by magnetic separation using human CD45 microbeads. CD45-depleted cells were plated on 96-well plates at a density of 0.5 cell/well in M199-EGM-2 (3:1, v/v) supplemented with 10% FBS and 1× antibiotic-antimycotic solution. The wells were analyzed for growing colonies twice weekly. Rapidly growing clones (2–3 colonies/sample) were harvested, resuspended in fresh growth medium, and plated on 0.1% gelatin-coated tissue culture dishes at a density of 5 × 10³ cells/cm². Cells were cultured under a humidified atmosphere of air/CO₂ (19:1) at 37°C for two to six 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 described previously (Anjos-Afonso and Bonnet, 2008) with minor modifications. In brief, Sca-1⁺CD31⁻ cells were plated onto a 0.1% gelatin A-coated two-chamber slide (Lab-Tek Chamber Slide; Thermo Fisher Scientific, Waltham, MA) at a density of 5 × 10³ cell/cm². Cells were grown in DMEM-LG medium supplemented with 10% FBS for 24 to 48 h to 80 to 90% confluence. 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 3-isobutyl-1-methylxanthine) were fixed for 30 min with 10% buffered formalin and stained with 0.21% Oil Red O (Sigma-Aldrich). 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-Aldrich).

Differentiation of human cardiac stromal cells toward cardiomyocyte lineage was conducted according to a previously described protocol (Smits et al., 2009). To induce cardiomyogenic differentiation of murine cells, cardiac stromal cells were pretreated 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 transforming growth factor β1, 100 μM ascorbic acid, 0.2% DMSO, and 10 ng/ml basic fibroblast growth factor for 3 weeks with medium changed every 3 days.

Flow Cytometry. All cells were analyzed either when freshly isolated from ventricles or after treatment of monolayer cultures with Accutase-Enzyme Cell Detachment Medium (eBioscience). Cells (~5 × 10⁵) 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 h 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 a Cytofix/Cytoperm kit (BD Biosciences), the permeabilized cells were stained for VEGF using rabbit anti-VEGF-A (Bioss USA, Woburn, MA), and FITC-conjugated donkey anti-rabbit antibody (BioLegend, San Diego, CA). Rabbit IgGs (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as an isotype control. Viable and nonviable cells were distinguished using LIVE/DEAD Fixable Blue Stain kit (Invitrogen). Data acquisition was performed using an LSRII flow cytometer (BD Biosciences), and the data were analyzed with WinList 5.0 software (Verity Software House, Inc., Topsham, ME). Antigen negativity was defined as having the same fluorescent intensity as the isotype control.

Real-Time 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 (Applied Biosystems, Foster City, CA). Primer pairs and 5'-carboxyfluorescein-labeled probes for human (A₁, Hs00181231; A_{2A}, Hs00169123; A_{2B}, Hs00386491; A₃, Hs00181223) and murine adenosine receptors (A₁, Mm01308023; A_{2A}, Mm00802075; A_{2B}, 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 1.

Transfections. The cDNA encoding the murine A_{2B} 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. Conditionally immortalized Sca-1⁺CD31⁻ cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN).

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

Analysis of Cytokine Secretion. Interleukin (IL)-6, CXCL1, IL-8, and VEGF concentrations in culture media were measured using enzyme-linked immunosorbent assay kits (R&D Systems).

Statistical Analysis. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and are presented as means ± S.E.M. 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. *p* < 0.05 was considered significant.

Results

Isolation and Characterization of Primary Cardiac Stromal Sca-1⁺CD31⁻ and Endothelial CD31⁺ Cells. To obtain the 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 Fig. 1A, left, this procedure resulted in enrichment of Sca-1⁺CD31⁻ cells up to approximately 80%. The 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% (Fig. 1A, right) and used next as a reference cell popu-

TABLE 1
Primers for RT-PCR used in this study

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mouse		
Bcrp1	CATCACATCACCTATCGAGTG	CATTAAGGCCAGGTTTCATG
Bmi1	CACCTAATGTGTGCTCCTGTGTG	GTGAACCTGGACATCACA
TERT	GCTGCAGAGCATTGGAGTCAG	CTTGGGGATGAAGCGCAGTCTG
Oct4	CAGCAGATCACTCACATCGCC	CAGAACCATACTCGAACCA
UTF1	CAAGAGTCCGAGCTGCCGCT	CGTGGGAAGAACTGAATCTG
GATA4	GGTAACCTCCAGCAATGCCACT	AGTGACAGGAGATGCATAGC
Nkx2.5	GTGCTCTCCTGCTTTCCCA	AGAAGAGCACGCGTGGCTTC
MEF2C	GGATGAATAATCGTAAGCCA	TGGTAAAGTAGGAGTTGCTAC
cTnT	GAGACAGACAGAGAGAGAGAA	TGCTGCTTGAACCTTTCTCTGC
α -Actin	GTGTGACATCGATATCCGCAA	TACTCTTGCTTGTGATCCAC
β -MHC	GAGCCGGCTGTGCTGTACAA	ATGTACTGATAGCGTTGTGTC
β -Actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
Human		
GATA-4	TCCAAACCAGAAAACGGAAG	CTGTGCCCGTAGTGAGATGA
Nkx2.5	GCCTTCTATCCACGTGCCTAC	GTCCGCCTCTGTCTTCTCCAG
MEF2C	AGATACCACACACACCACGCGCC	ATCCTTCAGAGAGTCGCATGCGCTT
cTnT	GTGGGAAGAGGCAGACTGAG	ATAGATGCTCTGCCACAGC
α -Actin	TCCTGATGCGCATTTTTATTC	AACACCACTGCTCTAGCCACG
β -MHC	GGAGGAGGACAGGAAAAACC	CTTGCGGAACCTGGACAGG
β -Actin	CGCCCCAGGCACACAGGGC	GGCTGGGGTGTGAAGGT

lation in the characterization of cardiac Sca-1⁺CD31⁻ stromal cells.

The collected Sca-1⁺CD31⁻ and CD31⁺ cell populations were plated and expanded to confluence in serum-free or endothelial growth media, respectively, as described under *Materials and 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 expression of CD34, CD117 (c-kit), and CD45 (Fig. 1B), displaying phenotypes previously reported for mesenchymal stem-like and endothelial cells, respectively (Lidington et al., 2002; Dominici et al., 2006; Tateishi et al., 2007; Ieronimakakis et al., 2008). 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, polycomb group protein Bmi1, and ATP-binding cassette transporter Bcrp1 compared with CD31⁺ cells (Fig. 1C). However, these cardiac stromal cells did not express the embryonic stem cell markers OCT4 or UTF1 (data not shown). Taken together, results for 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 A_{2B} receptors (Fig. 1D). Much lower levels of A_{2A} 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 A_{2B} receptor mRNA levels, although the expression of A_{2A} receptors was similar to that in Sca-1⁺CD31⁻ cells. As in Sca-1⁺CD31⁻ cells, no A₁ and A₃ receptor transcripts were detected in CD31⁺ cells.

A_{2A} and A_{2B} receptors are known to stimulate adenylate

cyclase via coupling to G_s proteins (Fredholm et al., 2001). Therefore, we measured cAMP accumulation as a way to determine whether expression of mRNA translates into the functional presence of adenosine receptors in Sca-1⁺CD31⁻ and CD31⁺ cardiac cell populations. As seen in Fig. 1E, the nonselective adenosine receptor agonist NECA (10 μ M) stimulated cAMP accumulation by 7.9 ± 0.7 -fold in Sca-1⁺CD31⁻ cells 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 whether 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 h in the absence or presence of 10 μ 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 (Fig. 1F, left). 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 (Fig. 1F, middle). In the absence of NECA, cardiac Sca-1⁺CD31⁻ stromal cells produced 5-fold higher levels of IL-6 compared with cardiac endothelial cells (Fig. 1F, right). IL-6 secretion from both cell types was augmented 1.7- to 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 with 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.

Fig. 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) and for Sca-1⁺ marker from the CD31⁻-depleted cells (left). Please see *Materials and 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 ± S.E.M. of three experiments. **D**, real-time RT-PCR analysis of mRNA encoding adenosine receptors in Sca-1⁺CD31⁻ and CD31⁺ cells. Values are averages of two experiments. **E**, cAMP accumulation in Sca-1⁺CD31⁻ and CD31⁺ cells in the absence (Basal) or presence of 10 μM NECA. Values are means ± S.E.M. of three experiments. **F**, VEGF, CXCL1, and IL-6 release from Sca-1⁺CD31⁻ and CD31⁺ cells in the absence (Basal) or presence of 10 μM NECA. Values are means ± S.E.M. of three experiments. Statistical differences: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, unpaired two-tailed t test; ns, nonsignificant difference compared with basal levels.

Flow cytometry analysis of CD45-negative cardiac cells showed that the proportion of Sca-1⁺CD31⁻ stromal cells was increased in the hearts that underwent myocardial infarction 1 week earlier compared with control sham-operated hearts (Fig. 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 1 week after coronary artery ligation (Fig. 2B). A considerable increase in the stromal to endothelial cell ratio

Fig. 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 A and total numbers of CD45-negative cells in each mouse heart. Values are means $\pm$ S.E.M. of four experiments. Statistical difference: *, $p < 0.05$; unpaired two-tailed t test, compared with control.

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 2 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 greater VEGF production in cardiac Sca-1⁺CD31⁻ stromal cells compared with 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 with 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 the ADA- and ADA+ experimental groups (Fig. 3).

Generation of Conditionally Immortalized Cardiac Sca-1⁺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 the Immortomouse (Charles River Laboratories International, Inc.) containing a gene encoding the thermolabile simian virus 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), to obtain sufficient Sca-1⁺CD31⁻ cells for analysis. Conditionally immortalized cells were grown for four to eight passages. Experiments were then performed with cells

Fig. 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 $\pm$ S.E.M. of four experiments. Statistical difference: **, $p < 0.01$; unpaired two-tailed t test; ns, nonsignificant difference compared with ADA+.

switched to their untransformed state by culture at 37°C in the absence of IFN- γ (i.e., nonpermissive conditions).

Figure 4A, top, shows phase-contrast and immunofluorescence (Fig. 4A, bottom) micrographs of Sca-1⁺CD31⁻ cells derived from Immortomouse hearts. As seen in Fig. 4A, bottom left, Sca-1⁺CD31⁻ cells cultured at 33°C with IFN- γ demonstrated abundant nuclear staining with anti-simian virus 40 large T antigen monoclonal antibody PAb101 (BD Pharmingen, San Diego, CA). However, after 48 h at 37°C in the absence of IFN- γ , tsA58 TAg expression was undetectable (faint nonspecific staining in Fig. 4A, bottom right). 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 whether the immortalized cells preserved the reported multipotent capacities of primary cardiac stromal cells (Matsuura et al., 2004; Tateishi et al., 2007), we deter-

Fig. 4. Characterization of conditionally immortalized cardiac Sca-1⁺CD31⁻ cells. **A**, phase-contrast (top) and tsA58 TAG immunofluorescent (bottom) micrographs of the expanded immortalized cardiac Sca-1⁺CD31⁻ cells cultured at 33°C in the presence of IFN-γ (left) or at 37°C in normal growth medium (right) 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 the respective adipogenic or osteogenic differentiating media (Diff) as described under *Materials and Methods*. Adipogenesis and osteogenesis were indicated by Oil Red O 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 under *Materials and Methods*. Values are averages of two experiments. cTnT, cardiac troponin T; β-MHC, β-myosin heavy chains.

mined 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 (Fig. 4 C; see also *Materials and Methods*). Up-regulation of several cardiac-specific genes, including transcription factors Nkx2.5, Mef2C, cardiac troponin T, 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 with that in cells cultured in a standard control medium for the same period (Fig. 4D; see also *Materials and Methods*). These changes in the phenotype of immortalized cardiac Sca-1⁺CD31⁻ cells under corresponding differentiating conditions are in close agreement with changes reported previously in primary cardiac stromal cells (Matsuura et al., 2004; Tateishi et al., 2007).

Role of A_{2B} Receptors in Adenosine-Dependent Secretion of Paracrine Factors from Cardiac Sca-1⁺CD31⁻ Cells. As with primary cells, conditionally immor-

talized WT cardiac Sca-1⁺CD31⁻ cells preferentially express mRNA encoding A_{2B} receptors. As expected, conditionally immortalized cells derived from A_{2B}KO mice did not express mRNA encoding A_{2B} receptors. Low levels of A_{2A} receptor mRNA were detected in both WT and A_{2B}KO Sca-1⁺CD31⁻ cells, whereas transcripts for A₁ and A₃ receptors were below detection levels (Fig. 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 A_{2B} receptors (Fig. 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 A_{2B}KO cells (Fig. 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 A_{2B}KO cells (Fig. 5D). Likewise, 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 A_{2B}KO cells (Fig. 5E). NECA effects were

Fig. 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 A_{2B}KO immortalized cardiac Sca-1⁺CD31⁻ cells. Values are averages of two experiments. **B**, effect of increasing NECA concentrations on cAMP accumulation in WT and A_{2B}KO immortalized cardiac Sca-1⁺CD31⁻ cells. Values are expressed as means $\pm$ S.E.M.; $n = 3$. Significant difference: **, $p < 0.01$; one-way ANOVA with Dunnett's post-test, compared with basal values. **C**, effect of increasing NECA concentrations on VEGF release from WT and A_{2B}KO immortalized cardiac Sca-1⁺CD31⁻ cells. Values are expressed as means $\pm$ S.E.M.; $n = 3$. Significant difference: **, $p < 0.01$; one-way ANOVA with Dunnett's post-test, compared with 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 μ M NECA. Values are means $\pm$ S.E.M. of three experiments. Statistical difference: ***, $p < 0.001$; unpaired two-tailed t test; ns, nonsignificant difference compared with 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 μ M NECA. Values are means $\pm$ S.E.M. of three experiments. Statistical difference: ***, $p < 0.001$; unpaired two-tailed t test; ns, nonsignificant difference compared with 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 the presence of 10 μ M NECA. Values are means $\pm$ S.E.M. of three experiments. Statistical difference: *, $p < 0.05$; unpaired two-tailed t test; ns, nonsignificant difference compared with basal levels.

rescued by transient expression of A_{2B} receptors in A_{2B}KO cells; transfection with a plasmid encoding mouse A_{2B} receptors but not with an empty vector enabled these cells to increase IL-6 release in response to NECA (Fig. 5F). Taken together, our results suggest that A_{2B} 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 whether 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 to 10% confluence on day 0 to 60 to 70% confluence on day 3. As with 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 (Fig. 6B). Incubation of human cardiac stromal cells in a cardiomyogenic 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 μ M NECA in a cardiomyogenic medium had no apparent effect on their expression (Fig. 6C).

Real-time RT-PCR analysis of human cardiac stromal cells revealed preferential expression of mRNA encoding A_{2B} receptors, with lower expression of A_{2A} receptors and no detectable levels of A₁ and A₃ receptor transcripts (Fig. 6D). Stimulation of adenosine receptors on human cardiac stromal cells with 10 μ M NECA for 6 h significantly increased release of IL-6, IL-8, and VEGF. Adenosine-dependent release of these factors was significantly inhibited by the selective A_{2B} receptor antagonist PSB-603 but not the selective A_{2A} receptor antagonist SCH52861 (Fig. 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_{2B} adenosine receptors can regulate paracrine functions of cardiac mesenchymal stem-like cells involved in regulation of angiogenesis. Our study revealed that cardiac Sca-1⁺CD31⁻ stromal cells predominantly express mRNA encoding the A_{2B} adenosine receptor subtype and considerably lower levels of A_{2A} adenosine receptor transcripts. Of importance, stimulation of adenosine receptors promoted release of the major proangiogenic factor VEGF from Sca-1⁺CD31⁻ stromal cells. Stimulation of adenosine receptors on Sca-1⁺CD31⁻ stromal cells also increased the release of CXCL1 and IL-6, factors known to promote angiogenesis (Strieter et al., 1995; Hernández-Rodríguez et al., 2003). Using conditionally immortalized mouse cardiac Sca-1⁺CD31⁻ stromal cell lines, we demonstrated that stimulation of adenosine receptors increased CXCL1, IL-6, and VEGF secretion in WT but not A_{2B}KO cells. Conversely, the loss of adenosine-dependent IL-6 secretion in A_{2B}KO cells was reversed by transient expression of recombinant A_{2B} receptors. Therefore, we concluded that A_{2B} receptors are essential for adenosine-dependent up-regulation of paracrine functions of Sca-1⁺CD31⁻ 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⁺CD31⁻ stromal cells but not CD31⁺ 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 ca-

Fig. 6. Characterization of human primary cardiac stromal cells. A, phase-contrast micrographs of human cardiac stromal cells immediately after replating of a single-cell-derived clone (d0, top) and 3 days later (d3, bottom). Scale bar, 100 μ 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 μ M NECA. Values are averages of two experiments. cTnT, cardiac troponin T; β -MHC, β -myosin heavy chains. D, real-time RT-PCR analysis of mRNA encoding adenosine receptors in human cardiac stromal cells. Values are averages of two experiments. E, IL-6, IL-8, and VEGF release from human cardiac stromal cells in the absence (Basal) or presence of 10 μ M NECA or in the presence of NECA and 100 nM SCH58261 or 100 nM PSB-603. Values are means $\pm$ S.E.M. of three experiments. Statistical difference: **, $p < 0.01$; ***, $p < 0.001$, compared with basal levels; †, $p < 0.05$; ††, $p < 0.001$; ns, nonsignificant difference compared with NECA-stimulated levels by one-way ANOVA with Bonferroni post-test.

pable of transdifferentiating 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 with cardiac CD31⁺ endothelial cells. The contribution of Sca-1⁺CD31⁺ stromal cells to VEGF production in the heart can be even greater after myocardial infarction. It has been shown previously that the 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 the 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 2 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 the 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 up-regulated VEGF production in retinal and skin microvascular endothelial cells but had no effect in human umbilical vein endothelial cells (Grant et al., 1999, 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 that 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 the 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 up-regulated 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 regard to 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, as 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 the 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 whether A₂ receptors remain functional in the isolated cardiac cells, our data do not necessarily imply that stimulation of all proangiogenic factors is cAMP-dependent. A_{2B} receptors have been linked to activation of not only G_s but also G_q 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 A_{2B} 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 A_{2B} 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). Which intracellular signaling pathways triggered by activation of A_{2B} receptors regulate production and release of specific proangiogenic factors from cardiac stromal cells remains to be elucidated.

Taken together, our results contribute to the growing evidence that A_{2B} receptors play an important role in neovascularization. We have shown previously that A_{2B} receptors up-regulate proangiogenic factors in retinal and skin endo-

thelial cells (Grant et al., 1999, 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 A_{2B} receptors, which are linked to up-regulation of proangiogenic factors and that similar A_{2B} receptor-dependent regulation exists in mesenchymal stem-like cells derived from the human heart. Thus, our study identified A_{2B} adenosine receptors on cardiac stromal cells as potential targets for up-regulation of proangiogenic factors in the heart. It remains to be determined whether stimulation and/or up-regulation 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 analytic 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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