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ORIGINAL ARTICLE

# Role of adenosine $A_{2B}$ receptor signaling in contribution of cardiac mesenchymal stem-like cells to myocardial scar formation

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**Abstract** Adenosine levels increase in ischemic hearts and contribute to the modulation of that pathological environment. We previously showed that $A_{2B}$ adenosine receptors on mouse cardiac $Sca1^+CD31^-$ mesenchymal stromal cells upregulate secretion of paracrine factors that may contribute to the improvement in cardiac recovery seen when these cells are transplanted in infarcted hearts. In this study, we tested the hypothesis that $A_{2B}$ receptor signaling regulates the transition of $Sca1^+CD31^-$ cells, which occurs after myocardial injury, into a myofibroblast phenotype that promotes myocardial repair and remodeling. In vitro, TGFβ1 induced the expression of the myofibroblast marker α-smooth muscle actin (αSMA) and increased collagen I generation in $Sca1^+CD31^-$ cells. Stimulation of $A_{2B}$ receptors attenuated TGFβ1-induced collagen I secretion but had no effect on αSMA expression. In vivo, myocardial infarction resulted in a rapid increase in the numbers of αSMA-positive cardiac stromal cells by day 5 followed by a gradual decline. Genetic deletion of $A_{2B}$ receptors had no effect on the initial accumulation of αSMA-expressing stromal cells but hastened their subsequent decline; the numbers of αSMA-positive cells including $Sca1^+CD31^-$ cells remained significantly higher in wild type compared with $A_{2B}$ knockout hearts. Thus, our study revealed a significant contribution of cardiac $Sca1^+CD31^-$ cells to the accumulation of αSMA-expressing cells after infarction and implicated $A_{2B}$ receptor signaling in regulation of myocardial repair and remodeling by delaying deactivation of these cells. It is plausible that this phenomenon may contribute to the beneficial effects of transplantation of these cells to the injured heart.

**Keywords** Adenosine · Receptor · Adenosine $A_{2B}$ · Mesenchymal stromal cells · Myofibroblasts · Myocardial infarction · Alpha-smooth muscle actin

## Introduction

Cardiac multipotent mesenchymal stem-like cells have been proposed as candidates for cell-based transplantation therapy to enhance tissue repair and functional recovery after myocardial infarction (MI) [1, 2]. In the mouse's heart, these cells are represented by a population of stromal cells characterized by cell-surface expression of stem cell antigen (Sca)-1 and absence of the endothelial cell marker CD31 [3–11]. Several groups, including our laboratory, have demonstrated that the delivery of cardiac $Sca1^+CD31^-$ cells to the injured heart attenuates the decline in cardiac function and the adverse remodeling in animal models of MI [6, 9, 11–13].

MI is known to increase interstitial adenosine concentrations to levels sufficient to engage all adenosine receptors, including the low-affinity $A_{2B}$ subtype [14–16]. The $A_{2B}$ receptor is expressed on mesenchymal stem/progenitor cells isolated from various tissues [10, 17–22] and represents the functionally predominant adenosine receptor subtype in cardiac $Sca1^+$ mesenchymal stem-like cells [10]. Importantly, we have recently demonstrated that $A_{2B}$ adenosine receptor

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signaling, linked to upregulation of paracrine factors in cardiac Sca1$^+$CD31$^-$ cells, is essential for the improvement of cardiac recovery resulting from transplantation of these cells to the injured heart [11]. On the other hand, we found no evidence that $A_{2B}$ receptors promote cardiomyogenic differentiation of cardiac mesenchymal stem-like cells [10].

Recent evidence suggests that cardiac mesenchymal stem-like cells respond to stimulation with TGFβ in vitro, or to myocardial injury in vivo, by displaying myofibroblast characteristics that include an increased synthesis of extracellular matrix (ECM) components and the expression of the contractile protein α-smooth muscle actin (αSMA) [23–25]. The rapid accumulation and activity of cardiac myofibroblasts early after myocardial injury is thought to be critical for proper scar formation [26]. Because the $A_{2B}$ adenosine receptor signaling is important for overall improvement of cardiac recovery seen after transplantation of cardiac Sca1$^+$CD31$^-$ cells to the infarcted heart [11], we hypothesized that it may also play a role in this novel aspect of mesenchymal stem-like cell function. In this study, we examined TGFβ-induced collagen I generation and the expression of αSMA by cardiac Sca1$^+$CD31$^-$ cells in vitro and evaluated the effect of stimulation of $A_{2B}$ adenosine receptors on these events. We also examined temporal changes in αSMA and collagen I expression in resident cardiac Sca1$^+$CD31$^-$ cells in vivo after performing experimental MI in $A_{2B}$ receptor knockout (KO) and wild-type (WT) mice.

## Materials and methods

*Reagents* Dulbecco's Modified Eagle Medium (DMEM) (high glucose) was purchased from Invitrogen Corporation (Carlsbad, CA). Porcine transforming growth factor β1 (TGFβ1) and mouse interferon γ (IFNγ) were purchased from R&D Systems (Minneapolis, MN). 5'-*N*-ethylcarboxamidoadenosine (NECA), fetal bovine serum (FBS), Antibiotic-Antimycotic solution, 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. $A_{2B}$KO mice were obtained from Deltagen (San Mateo, CA), and WT C57BL/6 mice were purchased from Harlan World Headquarters (Indianapolis, IN). All of the $A_{2B}$KO mice used in these studies were backcrossed to the C57BL/6 genetic background for more than 10 generations.

*Myocardial infarction* Surgical procedures to produce myocardial infarction in mice by permanent ligation of the left coronary artery were performed in the Cardiovascular Pathophysiology and Complications Core of the Vanderbilt University Mouse Metabolic Phenotyping Center as previously described [11].

*Mouse cardiac Sca1$^+$CD31$^-$ stromal cells* Conditionally immortalized cardiac Sca1$^+$CD31$^-$ stromal cell line was generated as described previously [10] from H-2K$^b$-tsA58 transgenic mice carrying a thermolabile T antigen. Cells were propagated on 0.1 % gelatin-coated tissue culture dishes in DMEM (high glucose) medium supplemented with 10 % FBS, 1X Antibiotic-Antimycotic solution, 2 mM glutamine, and 10 ng/ml of IFNγ under humidified atmosphere of air/$CO_2$ (19:1) at a low temperature (33 °C). Six days before experiments, cells were replated and cultured in the absence of IFNγ at a higher temperature (37 °C) to allow them to revert to their primary phenotype as described previously [10].

*Western blot analysis of collagen type I* To investigate the effect of TGFβ and NECA on the expression, secretion, and deposition of collagen I, Sca1$^+$CD31$^-$ cells were seeded on tissue culture-treated plates and cultured in DMEM medium supplemented with 10 % FBS, 1X Antibiotic-Antimycotic solution, and 2 mM glutamine for 24 h. Next day, culture media were changed with serum-free media supplemented with 1 ng/ml TGFβ1 and/or 30 μM NECA. After 48 h, conditioned media were collected, and total cell lysates were prepared using SDS lysis buffer. To prepare cell-free ECM, cells were lysed using 25 mM Tris-HCl (pH7.4)/150 mM NaCl/0.5 % Triton X-100/20 mM $NH_4OH$. Cell debris was washed using deionized water followed by PBS. Cell-free ECM remaining on tissue culture plates was collected using SDS sample buffer with β-mercaptoethanol. Equal volume of conditioned media and cell-free ECM and equal amount of total cell lysates from each treatment were resolved on 8 % SDS-PAGE. After blotting, the membrane was probed with antibodies raised against collagen type I (600-401-103; Rockland, Inc., Rockland, PA) or β-actin (Sigma) as a primary antibody and Streptavidin Poly-HRP Conjugate (Thermo Scientific, Inc., Waltham, MA) and HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX) as a secondary antibody. Blots were then developed using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and quantified by densitometry using ImageJ 1.45s software (National Institutes of Health, Bethesda, MD).

*Flow cytometry* All cells were analyzed either freshly isolated or after treatment with Accutase-Enzyme Cell Detachment Medium (eBioscience, San Diego, CA). Isolation of cardiac stromal cell populations was performed as previously

described [11]. In brief, both right and left ventricles were dissected from hearts, 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 $CaCl_2$) for 20 min at 37 °C. Filtered myocyte-free single-cell suspensions (~5x$10^5$) were washed and resuspended in 100 μl of PBS containing 0.5 % BSA and 2 mM EDTA (PBS/BSA/EDTA) and 2 μl of murine Fc block reagent (clone 2.4G, BD Biosciences, San Jose, CA). 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. Cell-surface antigens were stained with PE-conjugated anti-mouse CD31 or Sca-1 (eBioscience), PeCy7-conjugated Sca-1 or CD45, anti-CD105-APC or CD31-APC (Biolegend, San Diego, CA), and anti-CD45-V450 (BD Biosciences) antibodies. After treatment with Cytofix/Cytoperm kit (BD Biosciences), the permeabilized cells were stained for αSMA and collagen type I using monoclonal FITC-conjugated anti-αSMA (Sigma) and biotin-conjugated anti-collagen type I (600-401-103; Rockland, Inc., Rockland, PA) antibodies. Streptovidin-PeCy7 (eBioscience) or Streptavidin-Pacific Blue (Life Technologies) conjugates were used to detect biotin-conjugated antibodies. Mouse IgG2a-FITC-(Sigma) and biotin-conjugated rabbit whole IgG (Jackson ImmunoResearch, 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 (BD, Franklin Lakes, NJ), and the data were analyzed with WinList 5.0 software. Antigen negativity was defined as having the same fluorescent intensity as the isotype control.

*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 Bonferroni post-hoc tests. Comparisons between two groups were performed using two-tailed unpaired *t* tests. A $p$ value <0.05 was considered significant.

## Results

*Analysis of collagen I generation and the expression of αSMA by cardiac Sca1$^+$CD31$^-$ cells in vitro* We have previously shown that mouse cardiac Sca1$^+$CD31$^-$ cells, used in the current study, express predominantly the $A_{2B}$ subtype of adenosine receptors. Although low levels of $A_{2A}$ receptor transcripts were also detected, no evidence of their functional activity was found; only the non-selective adenosine agonist NECA, but not the selective agonist CGS 21680 stimulated cAMP accumulation in these cells [10]. To determine whether adenosine signaling in cardiac mesenchymal stem-like cells plays a role in the production of the common ECM component collagen I, we cultured mouse cardiac Sca1$^+$CD31$^-$ cells on uncoated plastic plates in the absence or presence of the stable adenosine analog NECA (30 μM) and in the absence or presence of the pro-fibrotic factor TGFβ1 (1 ng/ml) for 48 h. Figure 1a shows representative Western blots of conditioned media, cell-free ECM, and cell lysates analyzed with an antibody, which specifically recognizes the pro-α1 chain, the mature α1 chain, and the heterotrimer of type I collagen [27]. The expression of the 140 kDa pro-collagen α1(I) chains was clearly seen in cell lysates, whereas secretion of collagen I into media and its deposition on the plate surface were also evident by immunostaining of higher molecular weight bands representing heteromeric mature forms of type I collagen. Additional lower molecular weight bands seen only in conditioned media but not in extracellular matrix or cell lysates may represent accumulation of products of collagen I degradation. Stimulation of Sca1$^+$CD31$^-$ cells with TGFβ1 resulted in a several-fold increase in intracellular pro-collagen levels, accumulation of extracellular collagen I in conditioned media, and its deposition on the plate surface. Stimulation of adenosine receptors on Sca1$^+$CD31$^-$ cells with NECA, however, had much smaller effects on collagen I levels compared to the effects of TGFβ1. In the absence of TGFβ1, NECA had a tendency to increase intracellular pro-collagen levels and collagen I secretion by 1.4–1.6 fold, though these changes did not reach statistical significance (Fig. 1b). In contrast, stimulation of adenosine receptors in Sca1$^+$CD31$^-$ cells attenuated TGFβ-induced increase in collagen I levels in both conditioned media and ECM deposits by approximately 25 %, though only the changes in collagen I levels in conditioned media reached statistical significance. No difference in intracellular pro-collagen I levels was seen between cells stimulated with TGFβ1 in the absence and presence of NECA (Fig. 1a, b). These results suggest that stimulation of adenosine receptors with NECA in TGFβ-activated Sca1$^+$CD31$^-$ cells primarily inhibits collagen I release into conditioned medium. Conversely, in the absence of TGFβ1, NECA had a tendency to increase both intracellular pro-collagen I levels and collagen I release from non-activated Sca1$^+$CD31$^-$ cells in vitro. The effects of NECA on collagen I secretion were $A_{2B}$ receptor-specific because they were not observed in $A_{2B}$KO cells used as an off-target control (Fig. 1c, d).

In a separate set of experiments, we cultured mouse cardiac Sca1$^+$CD31$^-$ cells in the absence or presence of 30 μM NECA and in the absence or presence of 1 ng/ml TGFβ1 for 24 h and analyzed the expression of αSMA by fluorescence-activated cytometry sorting (FACS). Representative cytofluorographic dot plots of negative αSMA staining with an isotype-matched non-specific antibody are presented in Online Resource Fig. 1. In our cell culture conditions, even in the absence of TGFβ1

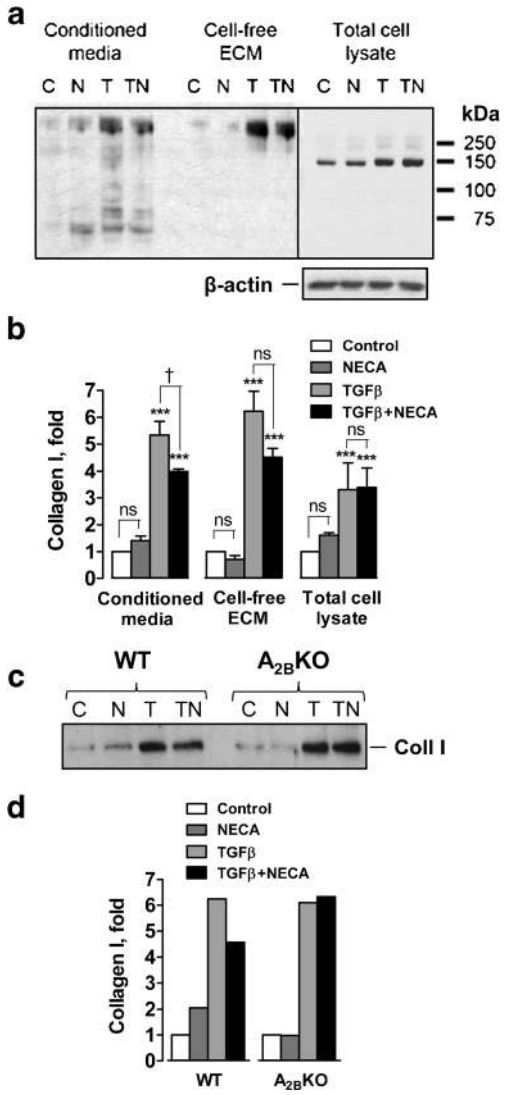


**Fig. 1** Stimulation of adenosine signaling with NECA attenuates TGFβ-induced secretion of collagen type I from cardiac Sca1$^+$CD31$^-$ cells in vitro. Representative Western blots (**a**) and densitometric analysis (**b**) of collagen type I (α1 chain) in conditioned media, cell-free extracellular matrix (ECM), and total cell lysates obtained after incubation of cardiac Sca1$^+$CD31$^-$ cells in the absence (C, N) or presence (T, TN) of 1 ng/ml TGFβ1 and in the absence (C, T) or presence (N, TN) of 10 μM NECA for 48 h. Molecular weight markers are shown on the far right, and β-actin was used as a loading control for analysis of cell lysates. Values are presented as means±SEM of three experiments. *Asterisks* indicate statistical difference (***$p<0.001$) compared to values obtained in the absence of TGFβ1, *dagger* indicates statistical difference ($^\dagger p<0.05$), and *ns* indicates non-significant differences compared to corresponding values obtained in the absence of NECA by one-way ANOVA with Bonferroni post-hoc tests. Western blots (**c**) and densitometric analysis (**d**) of collagen type I (Coll I) secretion into conditioned media from $A_{2B}$ knockout ($A_{2B}$KO) Sca1$^+$CD31$^-$ cells are shown as a negative control for $A_{2B}$ receptor signaling

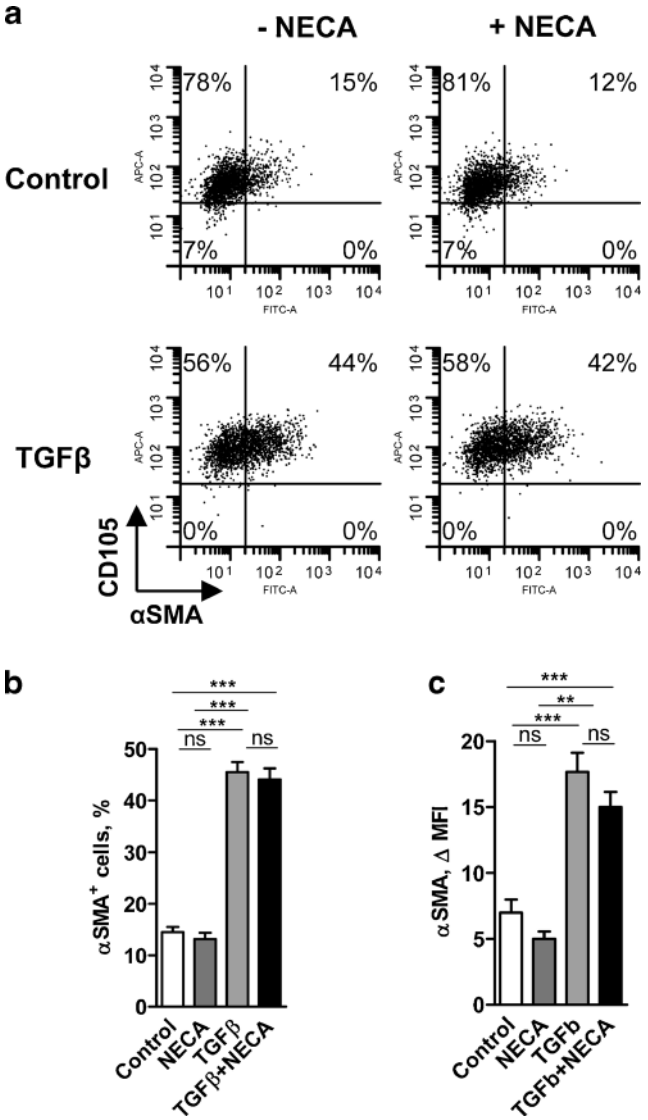


**Fig. 2** Stimulation of adenosine signaling with NECA has no effect on TGFβ-induced expression of αSMA in cardiac Sca1$^+$CD31$^-$ cells in vitro. Representative cytofluorographic dot plots (**a**) of intracellular αSMA staining in cardiac Sca1$^+$CD31$^-$ cells cultured in the absence (control) or presence (TGFβ) of 1 ng/ml TGFβ1 and in the absence (−NECA) or presence (+NECA) of 10 μM NECA for 24 h. The percentages of αSMA-positive cells (**b**) and their mean fluorescence intensity (ΔMFI) (**c**) are presented as means±SEM of three experiments. *Asterisks* indicate statistical differences (**$p<0.01$, ***$p<0.001$) and *ns* indicates non-significant differences analyzed by one-way ANOVA with Bonferroni post-hoc tests

and NECA, 14.5±1 % of total cell population expressed αSMA (Fig. 2). Stimulation of cells with TGFβ1 produced a three-fold increase in the proportion of αSMA-positive cells (Fig. 2b) and mean fluorescence intensity of αSMA staining (Fig. 2c). In contrast, stimulation of adenosine receptors with NECA had no significant effect on the expression of αSMA both in the presence or absence of TGFβ1.

*Analysis of the expression of αSMA and collagen I by cardiac Sca1$^+$CD31$^-$ cells in a mouse model of MI* TGFβ1 is only one of a multitude of factors that can promote stromal cell differentiation toward myofibroblast phenotype after myocardial injury. Obviously, stimulation of Sca1$^+$CD31$^-$ cells with

TGFβ1 in vitro cannot reproduce the complexity of their activation induced by MI in the heart. To gain insight into MI-induced dynamics of αSMA expression in a general stromal cell population and the potential role of $A_{2B}$ receptor signaling, we created experimental MI in $A_{2B}$KO and WT mice by permanent ligation of the left coronary artery. Cardiomyocyte-free cell suspensions were prepared from ventricles collected at different time points after MI induction. Gating strategy for the FACS analysis of αSMA expression in cardiac non-hematopoietic cells is presented in Online Resource Fig. 2. We found that only a small proportion of cells, representing approximately 2 % of non-hematopoietic cell population, expressed αSMA in non-infarcted hearts (d0, Fig. 3a). It is likely that this minor cell fraction consisted largely of vascular smooth muscle cells which express αSMA under normal conditions. MI induced a rapid rise in the proportion of αSMA-expressing cells in the population of stromal ($CD31^-$) but not endothelial ($CD31^+$) cells (Fig. 3a). An increase in numbers of αSMA-positive stromal cells, which peaked by post-MI day 5, was followed by gradual decline in their numbers over the next 16 days to nearly pre-infarct levels (Fig. 3b). Whereas no difference in numbers of αSMA-expressing cells was seen between $A_{2B}$KO and WT hearts during their rise on post-MI days 3 and 5, the numbers of αSMA-expressing cells remained significantly higher in WT compared with $A_{2B}$KO hearts during their decline (Fig. 3b). To determine if an increase in αSMA-expressing cell death rate in $A_{2B}$KO hearts could contribute to this phenomenon, we analyzed changes in cell viability produced by MI on days 3, 7, and 14 in myocyte-free cell suspensions obtained from $A_{2B}$KO and WT ventricles. As seen in Fig. 4a, MI produced an initial rise in proportion of non-viable cells by 13 % on day 3, which was reversed by days 7 and 14 comprising only 1.7–2.8 % of total cell populations. However, we found no significant differences between WT and $A_{2B}$KO hearts in MI-induced total cell death (Fig. 4a) or proportion of αSMA-expressing stromal cells within non-viable cell populations (Fig. 4b). Proportion of non-viable αSMA-expressing stromal cells in $A_{2B}$KO hearts tended to be even lower compared to WT hearts on post-MI days 7 and 14 suggesting that accelerated decline in αSMA-expressing stromal cell populations in $A_{2B}$KO versus WT hearts seen in Fig. 3 cannot be explained by their higher death rate.

Next, we selected post-MI days 7 and 14 to determine if there was also a difference in numbers of $Sca1^+CD31^-$ cells between $A_{2B}$KO and WT hearts. Representative cytofluorographic dot plots of negative $Sca1^+$ staining with an isotype-matched non-specific antibody are presented in Online Resource Fig. 3. Normal non-infarcted $A_{2B}$KO and WT hearts contained similar populations of $Sca1^+CD31^-$ cells (d0, Fig. 5). In agreement with previous reports [6, 10], MI induced a significant increase in $Sca1^+CD31^-$ cell numbers. We found that proportion of $Sca1^+CD31^-$

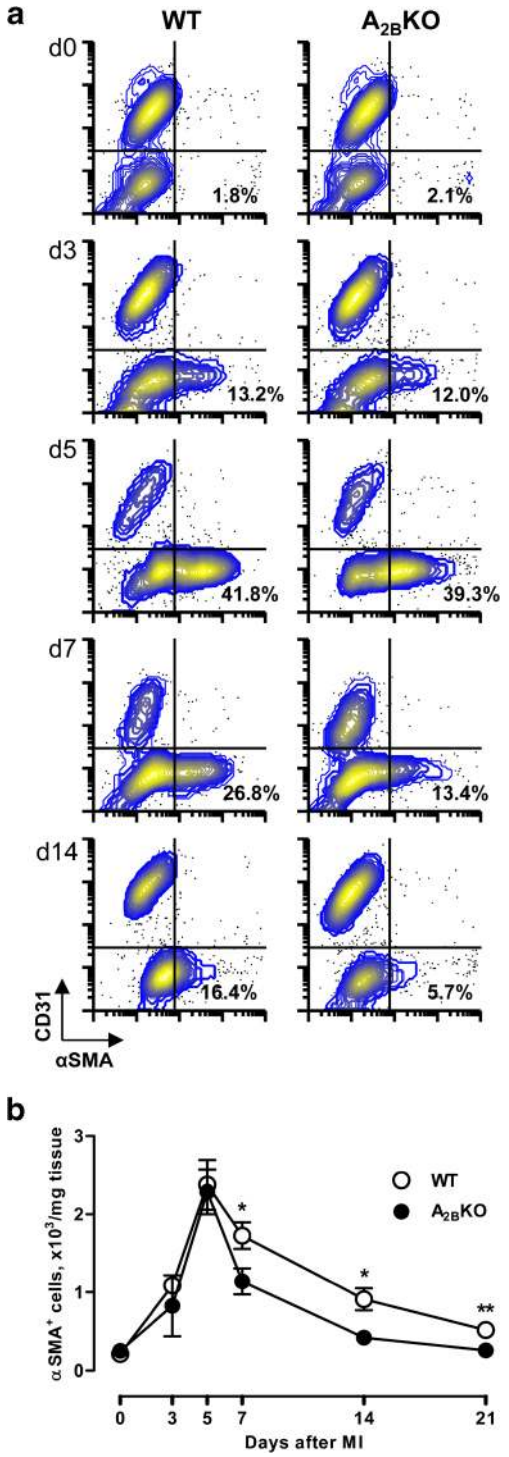


**Fig. 3** $A_{2B}$ receptor signaling controls deactivation of αSMA expression in total stromal cell population of infarcted ventricles. Representative cytofluorographic outlier contour plots of CD31 and αSMA expression (**a**) and the numbers of $CD31^-$ stromal cells expressing αSMA per milligram of tissue (**b**) in $CD45^-$ myocyte-free cell populations obtained from ventricles of WT and $A_{2B}$KO hearts before (d0) and on different days (d3–d21) after MI. Values are means±SEM of 4–6 animals in each group. *Asterisks* indicate statistical differences (*$p<0.05$, **$p<0.01$) between WT and $A_{2B}$KO values analyzed at each time point by unpaired two-tailed *t* test

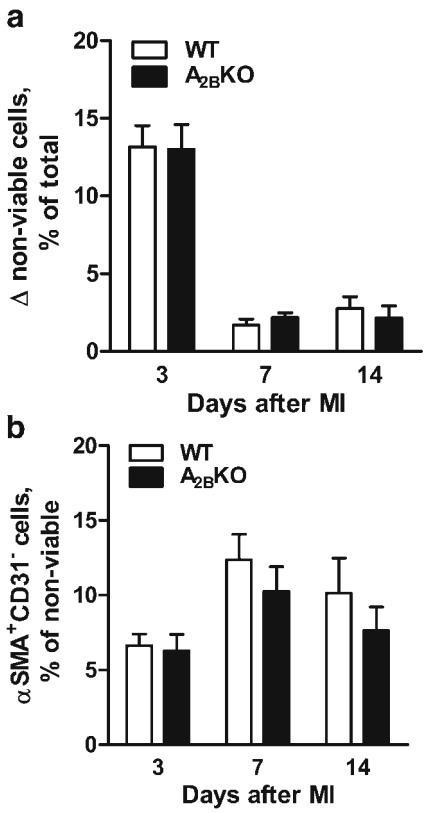


**Fig. 4** Comparative analysis of MI-induced changes in non-viable cell populations from WT and $A_{2B}$KO ventricles. MI-induced changes in total cell viability (**a**) determined as difference (Δ) between percentages of non-viable cells in total myocyte-free cell populations obtained from ventricles of WT and $A_{2B}$KO mice on days 3, 7, and 14 post-MI and percentages of non-viable cells in total myocyte-free cell populations obtained from non-infarcted ventricles of corresponding control mice. Percentages of α-SMA-expressing stromal ($CD31^-$) cells (**b**) in non-viable myocyte-free cell populations obtained from ventricles of WT and $A_{2B}$KO mice on days 3, 7, and 14 post-MI. Values are means± SEM of 4–6 animals in each group

cells in non-hematopoietic cell population (Fig 5b) and their numbers (Fig 5c) were significantly higher in WT compared with $A_{2B}$KO hearts on post-MI days 7 and 14. Finally, we analyzed the expression of αSMA and collagen 1 in the populations of $Sca1^+CD31^-$ cells obtained from $A_{2B}$KO and WT hearts. Representative cytofluorographic dot plots of negative αSMA and collagen I staining with control isotype-matched antibodies are presented in Online Resource Fig. 4. Before MI, the majority of the cardiac $Sca1^+CD31^-$ cell population (over 75 %) were double-negative for αSMA and collagen I expression; an even greater proportion of $Sca1^+CD31^-$ cells (>90 %) were negative for the expression of αSMA (d0, Fig. 6). MI induced a significant increase in proportion of $Sca1^+CD31^-$ cells expressing αSMA on post-MI days 7 and 14. Remarkably, the MI-induced changes in αSMA-expressing $Sca1^+CD31^-$ cell numbers (Fig. 6b, c) followed dynamics seen in total $Sca1^+CD31^-$ cell population (Fig. 5c), whereas the numbers of αSMA-negative $Sca1^+CD31^-$ cells were not significantly altered by MI (Fig. 6d, e). Thus, our data suggest that the increase in total $Sca1^+CD31^-$ cell population induced by MI occurs primarily due to generation of αSMA-expressing $Sca1^+CD31^-$ cells. Although some of the newly generated αSMA-expressing $Sca1^+CD31^-$ cells also expressed collagen I (Fig. 6b), a substantial number of αSMA-expressing $Sca1^+CD31^-$ remained collagen-negative (Fig. 6c). Again, the numbers of αSMA-expressing $Sca1^+CD31^-$ both negative and positive for the expression of collagen I were significantly higher in WT compared with $A_{2B}$KO hearts on post-MI days 7 and 14 (Fig 6b, c), whereas ablation of $A_{2B}$ receptor signaling in $A_{2B}$KO hearts had no significant effect on the numbers of αSMA-negative $Sca1^+CD31^-$ cells (Fig 6d, e). Taken together, our results indicate that $A_{2B}$ adenosine receptor signaling delays the reversal of $Sca1^+CD31^-$ cells from their differentiated myofibroblast-like state back to their resting state.

## Discussion

Cardiac mesenchymal stem-like cells received much attention lately due to their potential to transdifferentiate into various cell lineages. Under appropriate cell culture conditions, these cells can undergo cardiomyogenic, osteogenic, adipogenic, and chondrogenic differentiation [4, 10, 12, 23]. We have previously reported that stimulation of $A_{2B}$ receptors on cardiac $Sca1^+CD31^-$ cells had no effect on their in vitro differentiation toward cardiomyogenic, osteogenic, or adipogenic lineages [10]. In this study, we also found no significant effect of stimulation of $A_{2B}$ receptors on the expression of the myofibroblast marker αSMA in cardiac $Sca1^+CD31^-$ cultured in the presence of TGFβ. The steady-state levels of intracellular pro-collagen I were also unaffected, but TGFβ-induced secretion of collagen I into media and its deposition on the plate surface were attenuated by stimulation of adenosine receptors. Similar adenosine $A_{2B}$ receptor-dependent effects on collagen synthesis in vitro have been reported in serum-activated rat cardiac fibroblasts [28–32] suggesting that adenosine may play an anti-fibrotic role in the heart. In contrast, non-activated cardiac human fibroblasts treated with NECA demonstrated an increase in collagen secretion [33]. Furthermore, rat fibroblasts overexpressing $A_{2B}$ adenosine increased collagen synthesis in response to NECA [30]. In the current study, we also observed a tendency of non-activated $Sca1^+CD31^-$ cells to increase intracellular pro-collagen I levels and collagen I secretion in response to NECA. These effects were $A_{2B}$ receptor-specific because NECA had no effect on $A_{2B}$KO cells. Thus, $A_{2B}$ receptor-dependent regulation of collagen I production by cardiac

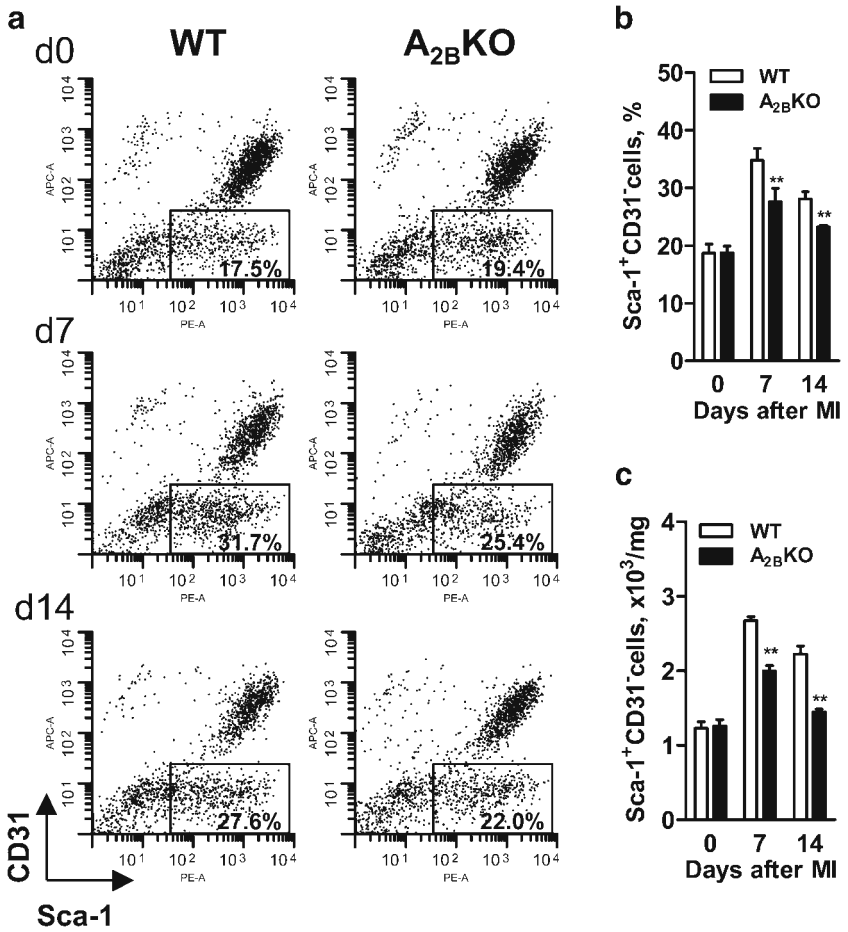


**Fig. 5** Comparative analysis of MI-induced changes in cardiac $Sca1^+CD31^-$ stromal cell populations of WT and $A_{2B}KO$ ventricles. Representative cytofluorographic dot plots (**a**) of CD31 and Sca1 cell-surface staining of $CD45^-$ myocyte-free cell populations obtained from ventricles of WT and $A_{2B}KO$ hearts before (d0) and on days 7 (d7) and 14 (d14) after MI. The percentages (**b**) and the numbers of $Sca1^+CD31^-$ per milligram of tissue (**c**) are presented as means±SEM of five animals in each group. *Asterisks* indicate statistical differences (*$p<0.05$, **$p<0.01$) between corresponding WT and $A_{2B}KO$ values analyzed by one-way ANOVA with Bonferroni post-hoc tests

$Sca1^+CD31^-$ stromal cells by adenosine in vitro is similar to that previously found in cardiac fibroblasts, which is highly dependent on culture conditions and particularly on their activation status. Importantly, treatment of $Sca1^+CD31^-$ stromal cells with NECA had no effect on the expression of αSMA in these cells regardless of their activation status.

In vivo, analysis of MI-induced αSMA expression in cardiac stromal cell population revealed a complex, dynamic, and time-dependent process. A rapid accumulation of αSMA-expressing cells was seen, reaching a maximum by post-MI day 5. The rapid accumulation of αSMA-expressing myofibroblasts in the injured area is believed to be important for scar contraction and proper wound healing [26]. Abrogation of $A_{2B}$ receptor signaling in $A_{2B}KO$ mice had no effect on the increase of αSMA-expressing cells. Thus, our in vivo results are in agreement with data obtained in cell culture experiments suggesting that $A_{2B}$ receptors play no role in generation of αSMA-expressing cells.

The deactivation of myofibroblasts is also believed to be critical for proper scar formation [26]. This process may involve apoptotic clearance [34] or de-differentiation of myofibroblasts accompanied by the loss of αSMA expression [35–37]. Indeed, after reaching their peak, the numbers of αSMA-expressing cells progressively declined over the next 16 days to nearly pre-infarct levels. It is during this phase when the difference between $A_{2B}KO$ and WT hearts becomes evident; the numbers of αSMA-expressing cells remained significantly higher in WT compared with $A_{2B}KO$ hearts. At the same time, we found no significant difference in proportion of non-viable αSMA-expressing cells between WT and $A_{2B}KO$ hearts. Taken together, our data suggest that $A_{2B}$ receptor signaling promotes retention of αSMA-expressing cells more likely by delaying myofibroblast de-differentiation rather than due to a decrease in their death rate. Although the precise mechanism of $A_{2B}$ receptor-dependent regulation of the deactivation of αSMA-expressing cells remains to be addressed in future cell-fate tracking experiments, our current study revealed a new level of complexity of adenosine actions in the heart. It also demonstrated that the effects of $A_{2B}$ receptors in the regulation of scar formation are time-dependent, and its role in this process may become more important at the healing phase of myocardial infarction.

The ramifications of altering the temporal regulation of αSMA-expressing cells on myocardial scar properties require further investigation. In support of beneficial effects of $A_{2B}$ receptor signaling in myocardial repair, it has been reported

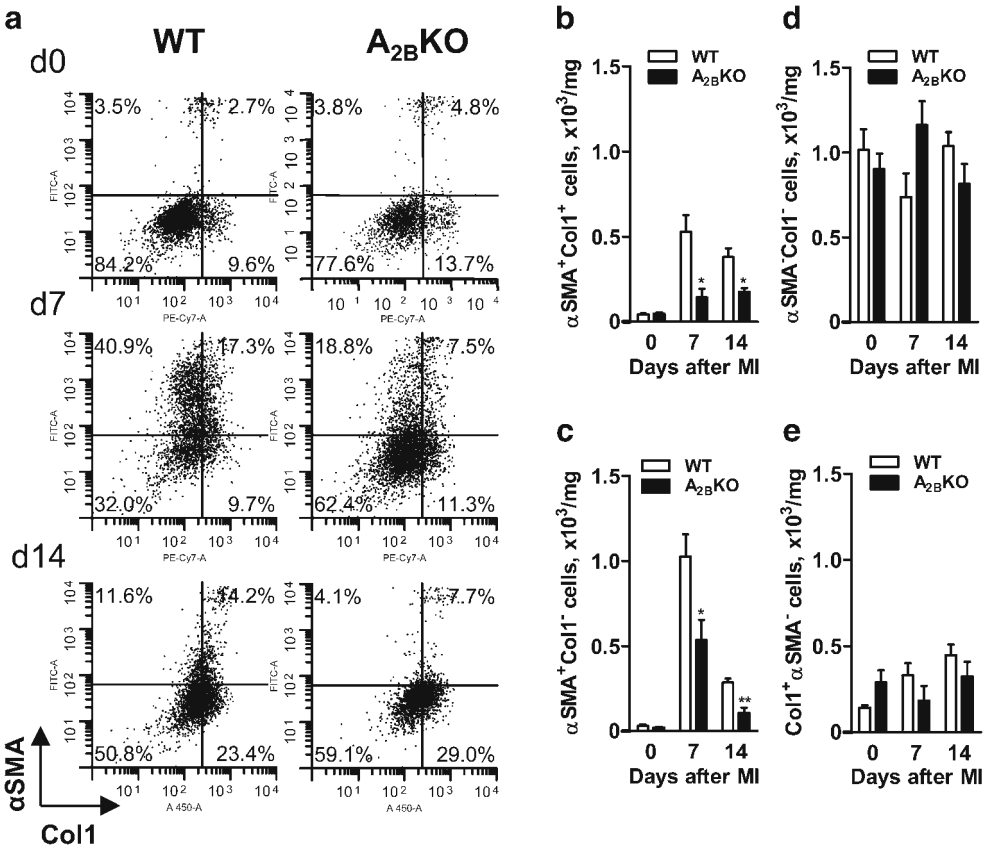


**Fig 6** Comparative analysis of MI-induced changes in αSMA and collagen I expression in cardiac Sca1$^+$CD31$^-$ stromal cell populations of WT and $A_{2B}$KO ventricles. Representative cytofluorographic dot plots (**a**) of intracellular αSMA and collagen I (Col1) expression in cardiac Sca1$^+$CD31$^-$ stromal cell populations obtained from ventricles of WT and $A_{2B}$KO hearts before (d0) and on days 7 (d7) and 14 (d14) after MI. The numbers of αSMA$^+$Col1$^+$ (**b**), αSMA$^+$Col1$^-$ (**c**), αSMA$^-$Col1$^-$ (**d**), and Col1$^+$αSMA$^-$ (**e**) cells per milligram of tissue are presented as means±SEM of 4 animals in each group. Asterisks indicate statistical differences (*$p$<0.05, **$p$<0.01) between corresponding WT and $A_{2B}$KO values analyzed by one-way ANOVA with Bonferroni post-hoc tests

that long-term stimulation of $A_{2B}$ receptors started one week after MI infarction significantly ameliorated adverse remodeling in the injured heart [38]. In contrast, activation of $A_{2B}$ receptor signaling at earlier stages of MI has been suggested to contribute to adverse remodeling by promoting an inflammatory response [39]. The retention of αSMA-expressing cells may help keep the injured tissue strong and contracted, thus preserving it from potential rupture. On the other hand, abnormal persistence of αSMA-expressing myofibroblasts may lead to interstitial fibrosis resulting in exaggerated mechanical stiffness, disorganized contraction, and worsening myocardial ischemia. In support of the latter concept, it has been reported that only WT but not $A_{2B}$KO mice developed reactive interstitial fibrosis in response to MI [40]. Furthermore, inhibition of $A_{2B}$ receptors with the selective antagonist GS-6201 reduced interstitial fibrosis in response to MI [41]. Although seemingly contradictory, these studies demonstrated a multifaceted and time-dependent regulation of MI-induced remodeling by $A_{2B}$ receptors. Due to well-known limitations of studies involving global knockout or systemic pharmacological modulation of $A_{2B}$ receptor signaling in such a complex process as MI, it is difficult to differentiate the direct effects of adenosine on stromal cells from indirect effects of other cells, e.g., invading leukocytes also known to be regulated by adenosine via $A_{2B}$ adenosine receptors [42, 43].

Despite these limitations, our study demonstrated for the first time that resident cardiac Sca1$^+$CD31$^-$ cells represent a substantial population of MI-induced αSMA-expressing cells and therefore can actively participate in scar formation. Furthermore, a proportion of Sca1$^+$CD31$^-$ in total non-hematopoietic cell population and their numbers increased nearly two-fold one week after MI and remained significantly higher in WT compared with $A_{2B}$KO hearts. Analysis of αSMA and collagen I expression suggested that almost all MI-induced changes in total Sca1$^+$CD31$^-$ cell population can be attributed to generation of αSMA-expressing Sca1$^+$CD31$^-$ cells comprised of both collagen-positive and collagen-negative populations. Like in general CD31$^-$ stromal cell population, the numbers of these cells remained significantly higher in WT compared with $A_{2B}$KO hearts indicating that their deactivation is regulated by the $A_{2B}$ receptor signaling. Thus, we conclude that $A_{2B}$ receptors play an important role the in regulation of dynamic changes in populations of α-SMA-expressing Sca1$^+$CD31$^-$ stromal cells induced by MI.

In summary, our study revealed a significant contribution of cardiac Sca1$^+$CD31$^-$ cells to the accumulation of αSMA-expressing cells after MI and implicated $A_{2B}$ receptor signaling in regulation of myocardial repair and remodeling by delaying deactivation of these cells. It is plausible that this phenomenon may also contribute in part to the beneficial

effects of mesenchymal stem-like cells seen after their transplantation to the injured heart.

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