

# Physiological Research Pre-Press Article

Expression of mRNA for adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> receptors in HL-60 cells:  
dependence on cell cycle phases

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Short title: Adenosine receptors and cell cycle

## Summary

The present studies investigated changes in expression of mRNA for adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> receptors in samples of HL-60 promyelocytic cells differing in the actual presence of cells in various phases of the cell cycle induced by the double thymidine block method. Real-time PCR technique was used for obtaining data on mRNA expression. Statistical analysis of the data revealed that the mRNA expression of adenosine A<sub>1</sub>, A<sub>2a</sub>, and A<sub>3</sub> receptors is dependent on the cell cycle phase. G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases were characterized by a higher mRNA expression of adenosine A<sub>1</sub> receptors and a lower one of adenosine A<sub>2a</sub> and A<sub>3</sub> receptors whereas the opposite was true for the S phase. Interestingly, expression of mRNA of the adenosine A<sub>2b</sub> receptors was independent on the cell cycle phase. The results indicate the plasticity of mRNA expression of adenosine receptors in the investigated promyelocytic cells and its interaction with physiological mechanisms of the cell cycle.

**Keywords:** adenosine receptors, messenger RNA expression, cell cycle, HL-60 promyelocytic cells

## Introduction

Adenosine, an ubiquitous purine nucleoside released into the extracellular environment from metabolically active or stressed cells, has been recognized to act as a paracrine regulator of many cellular functions including proliferation and differentiation (Abbracchio and Burnstock 1998, Poulsen and Quinn 1998, Schulte and Fredholm 2003). The regulatory function of extracellular adenosine is based on the activation of cell surface receptors, namely A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub>. Receptor activation can be achieved either non-selectively, by adenosine, or selectively, using various adenosine analogs (Jacobson 2002).

We have demonstrated that pharmacologically induced elevation of extracellular adenosine, i.e. non-selective activation of adenosine receptors, stimulates hematopoiesis in mice (for review, see Hofer and Pospíšil 2006, Hofer *et al.* 2011). Furthermore, we have aimed our effort at direct determination of mRNA expression for adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> receptors in four mouse hematopoietic precursor cell lines, namely granulopoietic/monocytopoietic, erythropoietic, B-lymphopoietic, and T-lymphopoietic ones. Similar studies were carried out with mouse RAW 264.7 macrophages taken as a model object representing the hematopoietic microenvironment. We have demonstrated that mRNA for all the four adenosine receptors is expressed in all cell types tested with the exception of the A<sub>1</sub> receptor in the RAW 264.7 macrophages (Štreitová *et al.* 2010 a, b). Thus, the ability

of important cells of the hematopoietic system to respond to activation by selective adenosine receptor agonists has been proven. Furthermore, we have used the model of hematopoietic suppression evoked by the cytotoxic drug 5-fluorouracil, killing predominantly cycling cells, and we have found that particularly administration of two tested selective adenosine receptor agonists, i.e. N<sup>6</sup>-cyclopentyladenosine (CPA) and N<sup>6</sup>-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), selective for A<sub>1</sub> and A<sub>3</sub> receptors, respectively, influences cycling of hematopoietic progenitor cells: activation of adenosine A<sub>1</sub> receptors has been found to inhibit whereas activation of adenosine A<sub>3</sub> receptors to stimulate proliferation in the compartments of both bone marrow progenitor and precursor cells (Pospíšil *et al.* 2004, 2005, Hofer *et al.* 2006, 2007, 2008).

Starting from the knowledge platform summarized above, we have addressed the problem of adenosine receptor expression and we have studied whether or not is the mRNA expression of individual adenosine receptors related to the varying functional state of the hematopoietic tissue induced by the variable presence of cells occurring in different phases of the cell cycle. HL-60 promyelocytic cells have been used as a model of the hematopoietic cell renewal system. The mRNA expression for individual adenosine receptors has been determined by the quantitative real-time PCR technique.

## **Materials and methods**

### **Cell System and Culture Conditions**

Human promyelocytic leukemic cell line HL-60 was obtained from the American Tissue Culture Collection (ATCC). The cells were cultivated in RPMI-1640 medium (Sebak, Aidenbach, Germany) supplemented with 10 % inactivated fetal bovine serum (Pan Biotech GmbH, Aidenbach, Germany), 1 mM sodium pyruvate (ICN Biomedicals, Costa Mesa, CA, USA), 100 µg/ml streptomycin (Gibco, Paisley, UK), 100 IU/ml penicillin (Gibco), 0.1 mg/ml gentamicin (PAN Systems, Nürnberg, Germany), 8 mM L-glutamine (Gibco), 1 % nonessential amino acids (ICN Biomedicals), 5mM HEPES (Serva, New York, NY, USA), and 50 µM 2-mercaptoethanol (Fluka, Seelz, Germany). The cells were kept at 37°C in a humidified atmosphere containing 95% air and 5 % CO<sub>2</sub>, transferred into a fresh medium twice a week and used for experiments during the exponential growth phase.

### Double Thymidine Block of HL-60 Cells

To obtain populations with varying percentages of cells in individual cell cycle phases, HL-60 cells were exposed to the double thymidine block. Stock solution of 200 mM thymidine (Sigma, St.Louis, MO, USA) in PBS was prepared in and used at a 1/50 dilution to produce a 4 mM thymidine solution. The cells in the exponential growth phase (analysed as sample 1) ( $5 \times 10^5$ ) were blocked for 18 h with 4 mM thymidine, released by washing out the thymidine once in phosphate-buffered saline (PBS, pH 7.4), and cultivated in fresh RPMI medium for 9 h. Then the cells were blocked again with 4 mM thymidine for 17 h to arrest a significant percentage of the cells at the beginning of S-phase. The arrest was then released by washing out thymidine and the cells were cultivated in fresh medium for 72 h. Further analyses were carried out at hours 1 (sample 2), 2 (sample 3), 3 (sample 4), 6 (sample 5), 24 (sample 6), 48 (sample 7) and 72 (sample 8). Relative representation of the cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M cell cycle phases in the cell samples is shown in Table 1.

### Cell Cycle Studies

The sampled cells were collected by centrifugation, washed once in PBS and suspended for 12 hours in 70 % ethanol for cell fixation at the temperature of 4 °C. The fixed cells were again collected by centrifugation, ethanol was aspirated off and the cells were resuspended in a staining buffer containing 5mg/ml propidium iodide, 1M TRIS (Roth, Karlsruhe, Germany) (pH 8), 10mg/ml RNase (Sigma) and 10 % Triton X-100 (AppliChem, Darmstadt, Germany). After 30 min of incubation at 37 °C, samples were analyzed with a FACScan flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser (excitation at 488 nm). To estimate the percentage of cells in each phase of cell cycle (G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M), histogram analyses of relative DNA contents were carried out with Mod Fit LT 2,0 software (VERITY, Software House, Inc., Topsham, ME, USA).

### Isolation of Total RNA

In all samples (1 to 8), isolation of total RNA of the cells was carried out using the High Pure Isolation Kit (Roche, Mannheim, Germany). RNA concentration and purity were measured on Nano-Drop ND1000 Spectrophotometer (Thermo Scientific, USA). The 260/280 ratio was not less than 1.8 for each RNA sample. Total RNA was stored at -80 °C prior to cDNA synthesis.

### Reverse Transcription of total RNA to cDNA

The subsequent cDNA synthesis for a two-step real-time polymerase chain reaction (RT-PCR) was carried out with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). One  $\mu\text{g}$  of RNA template was used per each RNA-to-cDNA reaction. The templates were mixed with anchored-oligo(dT18) primers (2.5  $\mu\text{M}$ ) and random hexamer primers (60  $\mu\text{M}$ ). The samples were placed in DNA Engine Peltier thermal cycler (BioRad, USA) and incubated for 10 min at 25°C, followed by 30 min at 55°C, and at the end heated to 85°C for 5 min. Synthesized cDNA was stored at -20°C.

### Quantitative Real-Time PCR (RT-PCR)

Using FastStart SYBR Green Master (Roche) in combination with appropriate PCR primers, RT-PCR detection and quantification of DNA sequences for A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> subtypes of adenosine receptors were performed. Sequences of primers (see Štreitová *et al.* 2010a) were taken over from Ashton *et al.* (2003) and Overbergh *et al.* (1999) and the primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic). RT-PCR was done in duplicates for each adenosine receptor primer on Rotor-Gene 6000 (Corbett Research, Sydney, Australia). The final reaction volume (20  $\mu\text{l}$ ) included 10  $\mu\text{l}$  of FastStart SYBRGreen Master Mix, 2.5  $\mu\text{l}$  of cDNA, and 300 nM concentration of each primer. The first reaction cycle was carried out at 95°C for 10 min and was followed by 40 cycles, each of which consisted in 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. To calculate relative gene expression, the delta-delta Ct method based on the difference of threshold cycles (Ct) of the target gene and the  $\beta$ -actin sequence was used. A twofold increase in PCR products per cycle was assumed. Any receptor was taken for relative quantification if the threshold cycle number was less than 36. If the threshold cycle number was greater than 36, the receptor mRNA was considered to be present in minimal quantities and relative quantification was not performed. A gene was considered not to be expressed if no amplification was detected by cycle 40.

### Statistical Analysis

Primary data were obtained on the basis of two repeated experiments. In each experiment, two determinations of the mRNA expression from each sample were performed. Arithmetic means were calculated from the two repeated determinations in each sample and then calculations of arithmetic means from both experiments were carried out. Thus, a single value representing the expression of a particular receptor in each sample was obtained. This aggregation of

primary data was carefully checked and it was proved not to reduce overall experimental variance.

Mutual association of examined parameters was evaluated on the basis of rank Spearman's non-parametric correlation and its statistical significance. Variance associated with the estimated correlations was assessed and the statistical significance of mutual differences between correlation coefficients was evaluated by a standard test based on normally distributed test statistics  $Z$  (Borkowf 2000, Cornbleet and Shea 1978).

The value  $p \leq 0.05$  was considered to be a boundary of statistical significance in all performed analyses. The analyses were carried out after removal of outliers (4.2 %) identified by Dixon's test (Dean and Dixon 1951, Rorabacher 1991). All analyses were performed using the SPSS 18.0.3. software (IBM Corporation, 2010).

## Results

The experimental protocol employing the promyelocytic HL-60 cell line was based on investigations of relationships between mRNA expression of individual adenosine receptors and the presence of cells occurring in individual phases of the cell cycle, i.e.  $G_0/G_1$ , S, and  $G_2/M$ . These analyses were performed separately for each receptor type and each cell cycle phase. Because of the four analysed adenosine receptors and the three investigated cell cycle phases, twelve correlations in total have been computed. Spearman's correlation coefficients  $r_s$  were used as a robust measure of association between given adenosine receptor mRNA expression and given cell cycle phase. Thus, a positive value of  $r_s$  indicates that the given phase of the cell cycle is linked with a higher production of the given receptor mRNA. On the contrary, a negative value of  $r_s$  indicates that the given cell cycle phase is linked with a lower level of the given receptor mRNA production. Results of the experiments are presented in Figs. 1 – 3 which characterize the tested functional dependences by regression lines and Spearman's correlation coefficients ( $r_s$ ) with corresponding statistical significances ( $p$ ).

The first distinct phenomenon of the results is the evidence that the mRNA expression of the adenosine  $A_{2b}$  receptors is inert to the structure of cell cycle phases in the individual samples: in all samples it reaches nearly the same level. The experimental variability associated with the mRNA expression of the adenosine  $A_{2b}$  receptors is very low, at the level of random error of fluctuations and no significant correlation occurred. This is in contrast to the experimental variability observed when analysing the expression of mRNA of all other adenosine receptor subtypes (see Figs. 1-3).

Concerning the cells in the  $G_0/G_1$  phase of the cell cycle (Fig. 1), the following observations are worth mentioning: There is a positive and significant correlation for the expression of adenosine  $A_1$  receptor mRNA, a negative and significant correlation for the expression of adenosine  $A_{2a}$  receptor mRNA, as well as a negative correlation ( $p=0.052$ ) for the adenosine  $A_3$  receptor mRNA. These findings suggest a higher level of mRNA expression of the  $A_1$  receptors and a lower one of the  $A_{2a}$  and  $A_3$  receptors in cells occurring in the  $G_0/G_1$  phase of the cell cycle.

Differently pronounced results have been found by calculations of association between the mRNA expression of individual adenosine receptors and the presence of the cells in the S phase of the cell cycle (Fig. 2). Here, the negative  $r_s$  for adenosine  $A_1$  receptor mRNA and positive  $r_s$  for mRNA of adenosine  $A_{2a}$  and  $A_3$  receptors occur, even if not attaining statistical significance. These findings seem to indicate that the manifestations of the mRNA expression of the adenosine  $A_1$ ,  $A_{2a}$ , and  $A_3$  receptors in the S phase of the cell cycle are opposite to those obtained in the  $G_0/G_1$  phase in terms of negativity or positivity of  $r_s$ . For confirmation of this phenomenon, correlation coefficients between the mRNA expression of the three adenosine receptors and the presence of the cells in the cell cycle phases  $G_0/G_1$  and S have been compared and the results are presented in Table 2. It is apparent that the mRNA expression of adenosine  $A_1$ ,  $A_{2a}$ , and  $A_3$  receptors differs significantly between the  $G_0/G_1$  and the S phases of the cell cycle. For the  $G_0/G_1$  phase an increased mRNA expression of the adenosine  $A_1$  receptor is typical whereas in the S phase an expression of the adenosine  $A_{2a}$  and  $A_3$  receptors predominates.

Data given in Fig. 3 demonstrate the dependence of the mRNA expression of adenosine receptors on the presence of the cells in the  $G_2/M$  phase of the cell cycle. Here, a negative statistically significant  $r_s$  for the mRNA expression of the  $A_{2a}$  receptor dominates. This situation is reflected also by the significant difference of correlation coefficients for the expression of mRNA for the adenosine  $A_{2a}$  receptor between the S and  $G_2/M$  phases, as shown in Table 2.

Additional statistical processing of the data consisted in computing of correlation coefficients representing the mutual relations in mRNA expression in pairs of adenosine receptors ( $A_1 - A_{2a}$ ,  $A_1 - A_3$ ,  $A_{2a} - A_3$ ) when using data from all cell cycle phases taken as a whole. The results are given in Table 3 and show a statistically significant positive correlation between the mRNA expression of adenosine  $A_{2a}$  and  $A_3$  receptors. There is also a negative correlation in receptor mRNA expression between  $A_1$  and  $A_{2a}$  receptors, as well as between that of  $A_1$  and  $A_3$  receptors, but without statistical significance.

## Discussion

To our knowledge, the obtained data present the first study investigating the expression of mRNAs of adenosine receptors in different phases of the cell cycle. Even if the rate of mRNA expression needs not be fully reflected at the corresponding protein levels, the presented data suggest at least tendencies for the synthesis of functional adenosine receptors and allows to formulate certain general conclusions.

The experiments performed on HL-60 promyelocytic cells suggest a diversity of mRNA expression of individual adenosine receptors in different phases of the cell cycle. The preparatory  $G_0/G_1$  phase, as well as the  $G_2/M$  phase, are positively linked to the mRNA expression of the  $A_1$  receptors and negatively to that of the  $A_{2a}$  and  $A_3$  adenosine receptors. On the contrary, the synthetic S phase is characterized by the decrease of the mRNA expression of the  $A_1$  adenosine receptors and the increase of that of the  $A_{2a}$  and  $A_3$  adenosine receptors. These findings might be analogical to our earlier considerations ensuing from *in vivo* experiments. We have observed positive effects of an adenosine  $A_3$  receptor agonist on cycling of hematopoietic cells (Pospíšil *et al.* 2004, Hofer *et al.* 2006, 2007, 2008), as well as an inhibitory action of an agonist of adenosine  $A_1$  receptors on the proliferation of cells in the murine hematopoietic system *in vivo* (Pospíšil *et al.* 2004, 2005, Hofer *et al.* 2008). Of particular interest are our findings in mice whose hematopoiesis was suppressed by 5-fluorouracil (Hofer *et al.* 2008). In these mice, the proliferation-stimulating effects of the adenosine  $A_3$  receptor agonist were most pronounced in the phase of maximum cell depletion when maximum cell proliferation was to be expected. On the other hand, the proliferation-inhibiting action of the adenosine  $A_1$  receptor agonist was most pronounced in the phase of maximum cell regeneration when a feedback suppressing the overshooting cell proliferation was needed (Hofer *et al.* 2008). Taking into account these findings and considerations, the data presented here on expression of mRNA of adenosine  $A_1$  and  $A_3$  receptors, which varies between the  $G_0/G_1$ , S, and  $G_2/M$  phases of the cell cycle, might be a further evidence of the regulatory and homeostatic role of these two adenosine receptors.

Although an analogical behavior of premyelocytic HL-60 cells with hematopoietic progenitor and precursor cells *in vivo*, as discussed in the previous paragraph, can be expected, it cannot be completely excluded that the very process of malignization of the HL-60 cells could influence the mRNA expression of adenosine receptors. Therefore, certain caution should accompany the interpretation of the results obtained.

The opposite role of the adenosine  $A_1$  and  $A_3$  receptors in the regulation of cell cycling might be surprising because both these receptors are known to inhibit adenylyl cyclase.



However, they differ in their coupling to different G proteins (Fredholm *et al.* 2000) and to phospholipases C and D (Parsons *et al.* 2000). Another example of opposite effects of the two adenosine receptors was shown in experiments of Lee and Emala (2000) demonstrating that adenosine A<sub>1</sub> receptor activation reduces, while adenosine A<sub>3</sub> receptor activation worsens renal ischemia-reperfusion injury.

An interesting phenomenon of the presented experiments is the behavior of the expression of mRNA for adenosine A<sub>2a</sub> receptors which significantly positively correlates with that for adenosine A<sub>3</sub> receptors. Activation of adenosine A<sub>2a</sub> receptors inhibits inflammatory processes in neutrophils, platelets, macrophages, and T cells (Linden 2001). Our earlier experiments investigating effects of the agonist of these receptors exhibited no significant effects on the cycling of haematopoietic cells (Pospíšil *et al.* 2004). Thus, the role of the adenosine A<sub>2a</sub> receptors in hematopoiesis and the relationships between the adenosine A<sub>2a</sub> and A<sub>3</sub> receptor mRNA expression deserve further attention.

Worth noting is also the evidence of the independence of the expression of mRNA of adenosine A<sub>2b</sub> receptors on the different phases of the cell cycle. The reason might be the low affinity of these receptors towards the endogenous agonist adenosine (Fredholm 2007) and thus operation of adenosine A<sub>2b</sub> receptors may be important only under pathophysiological conditions.

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## Legends to Figures

Fig. 1. Dependence of expression of mRNA for adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> receptors on the per cent proportion of HL-60 cells in the G<sub>0</sub>/G<sub>1</sub> cell cycle phase, as presented in Table 1.  $r_s$  – Spearman's correlation coefficient;  $p$  – statistical significance of  $r_s$ .

Fig. 2. Dependence of expression of mRNA for adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> receptors on the per cent proportion of HL-60 cells in the S cell cycle phase, as presented in Table 1.  $r_s$  – Spearman's correlation coefficient;  $p$  – statistical significance of  $r_s$ .

Fig. 3. Dependence of expression of mRNA for adenosine A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> receptors on the per cent proportion of HL-60 cells in the G<sub>2</sub>/M cell cycle phase, as presented in Table.  $r_s$  – Spearman's correlation coefficient;  $p$  – statistical significance of  $r_s$ .

**Table 1.** Per cent representation of G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M cell cycle phases in individual cell populations (samples described in the text)

Sample	G <sub>0</sub> /G <sub>1</sub> phase	S phase	G <sub>2</sub> /M phase
1	67.0	32.5	0.5
2	35.8	60.3	3.9
3	36.0	61.7	2.3
4	37.9	60.2	1.9
5	39.6	40.6	19.8
6	41.7	40.3	18.0
7	54.0	35.9	10.1
8	69.2	25.0	5.8

**Table 2.** Comparison of correlation coefficients  $r_s$  between cell cycle phases  $G_0/G_1$  and S, and between cell cycle phases S and  $G_2/M$ , for mRNA expression of adenosine  $A_1$ ,  $A_{2a}$ , and  $A_3$  receptors

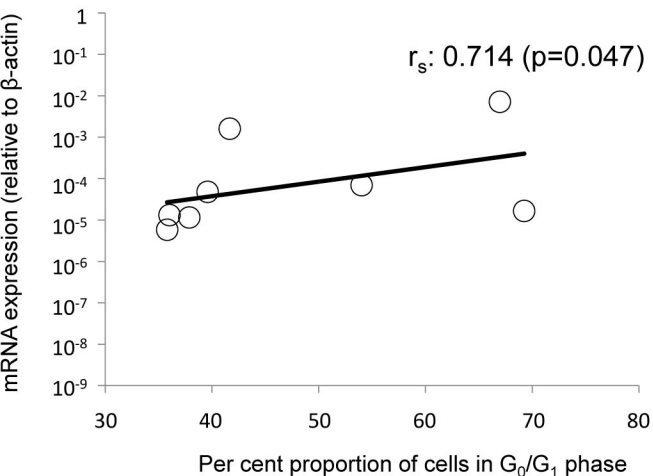
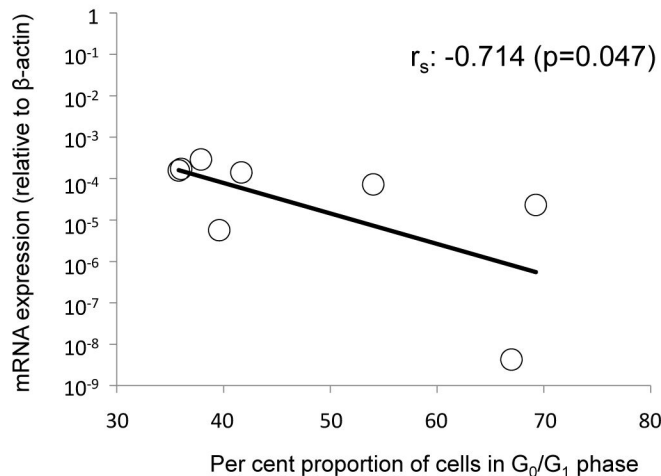
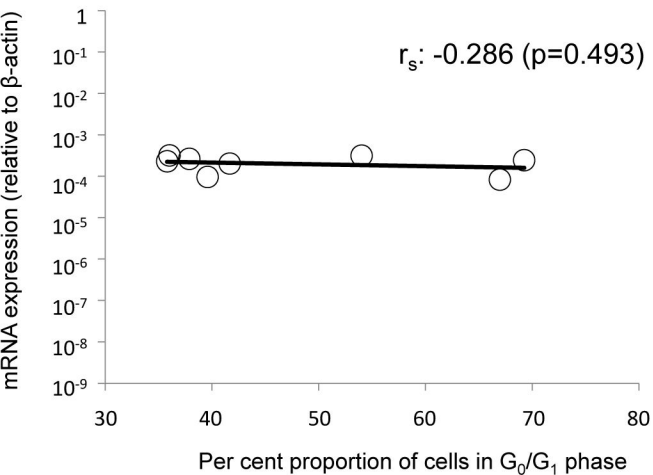
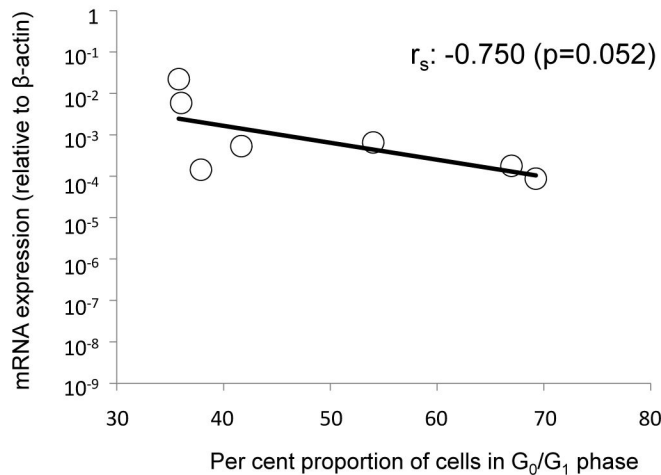
Receptor type	$G_0/G_1$ phase ( $r_s$ )	S phase ( $r_s$ )	$G_2/M$ phase ( $r_s$ )	Comparison of $r_s$ ( $G_0/G_1$ vs. S)	Comparison of $r_s$ (S vs. $G_2/M$ )
$A_1$	0.714	-0.667	0.190	p=0.021	p=0.141
$A_{2a}$	-0.714	0.679	-0.857	p=0.027	p=0.014
$A_3$	-0.750	0.714	-0.238	p=0.025	p=0.118

p – value of statistical significance of comparison of correlation coefficients  $r_s$  between the compared cell cycle phases.

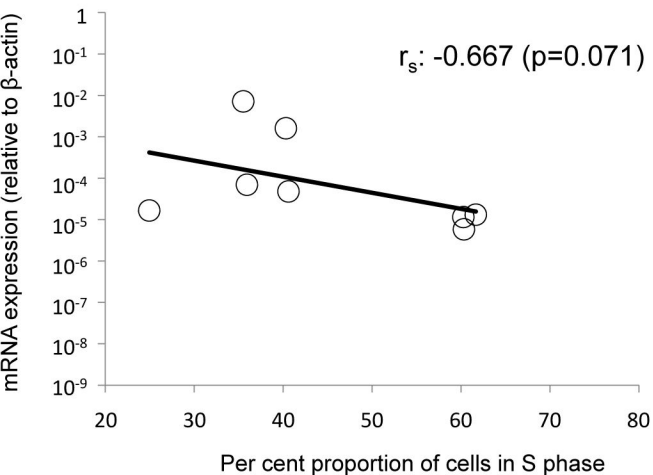
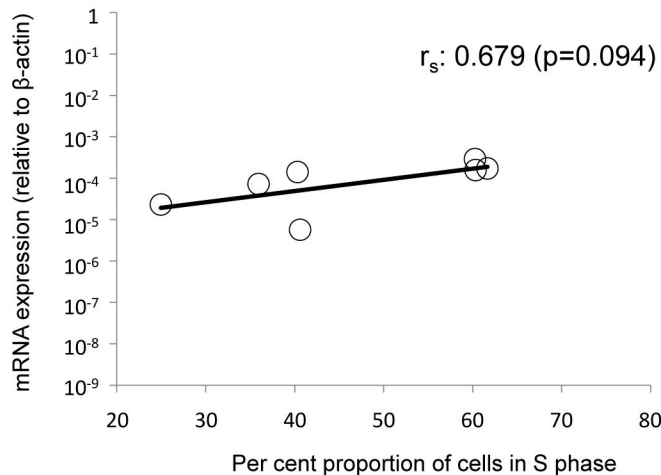
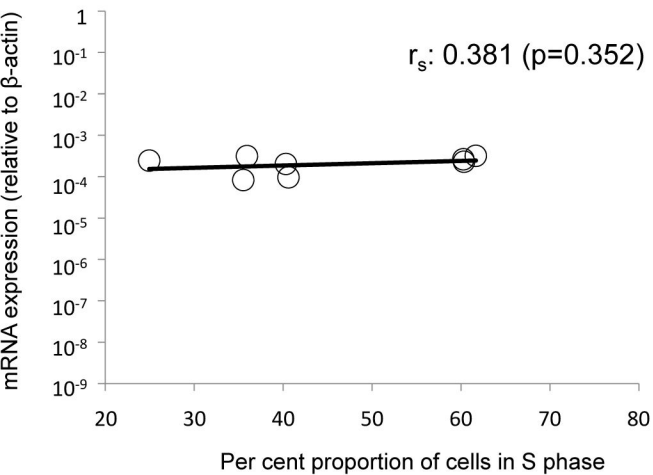
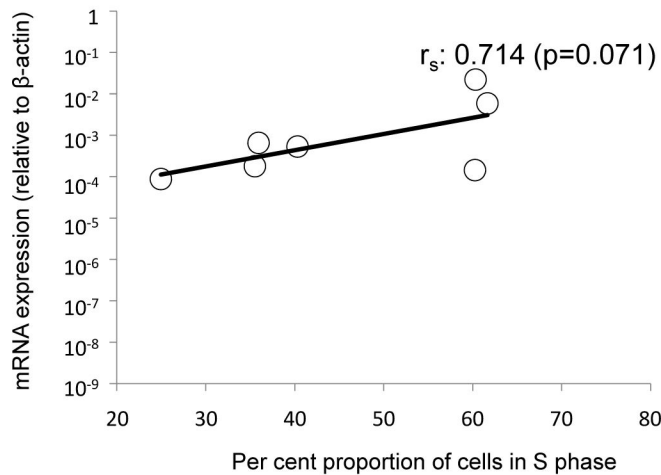
Table 3. Relations of mRNA expression indices between individual pairs of adenosine A<sub>1</sub>, A<sub>2a</sub>, and A<sub>3</sub> receptors

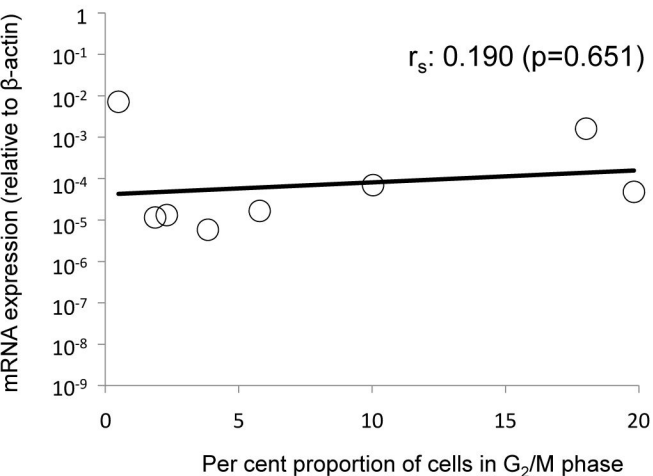
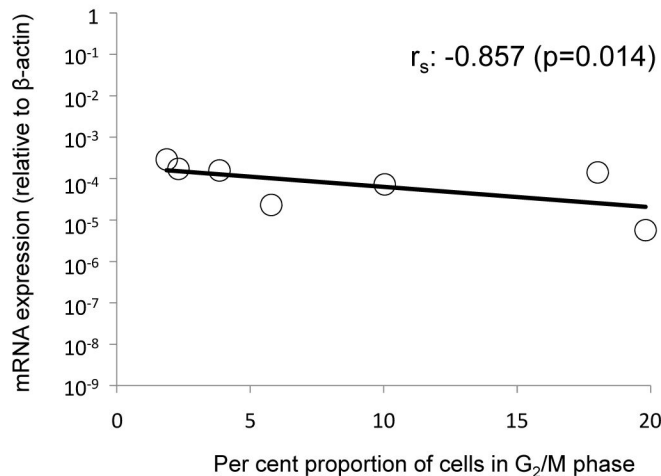
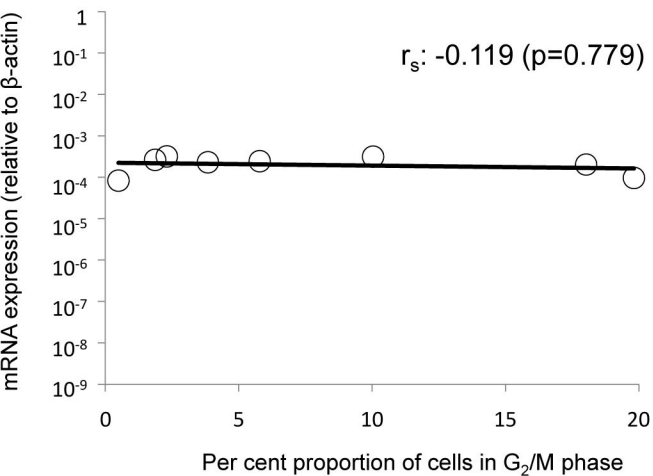
Receptor type	A <sub>1</sub>		A <sub>2a</sub>		A <sub>3</sub>	
	r <sub>s</sub>	p	r <sub>s</sub>	p	r <sub>s</sub>	p
A <sub>1</sub>	-	-	-0.143	0.760	-0.393	0.383
A <sub>2a</sub>	-0.143	0.760	-	-	0.786	0.036
A <sub>3</sub>	-0.393	0.383	0.786	0.036	-	-

r<sub>s</sub> – correlation coefficients; p – values of statistical significance of the pertinent r<sub>s</sub>

**a) A<sub>1</sub> receptor****b) A<sub>2a</sub> receptor****c) A<sub>2b</sub> receptor****d) A<sub>3</sub> receptor**



**a) A<sub>1</sub> receptor****b) A<sub>2a</sub> receptor****c) A<sub>2b</sub> receptor****d) A<sub>3</sub> receptor**

**a) A<sub>1</sub> receptor****b) A<sub>2a</sub> receptor****c) A<sub>2b</sub> receptor****d) A<sub>3</sub> receptor**