Downregulation of A_1 and A_{2B} adenosine receptors in human trisomy 21 mesenchymal cells from first-trimester chorionic villi

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ABSTRACT

Human reproduction is complex and prone to failure. Though causes of miscarriage remain unclear, adenosine, a proangiogenic nucleoside, may help determine pregnancy outcome. Although adenosine receptor (AR) expression has been characterized in euploid pregnancies, no information is available for aneuploidies, which, as prone to spontaneous abortion (SA), are a potential model for shedding light on the mechanism regulating this event. AR expression was investigated in 71 first-trimester chorionic villi (CV) samples and cultured mesenchymal cells (MC) from euploid and TR21 pregnancies, one of the most frequent autosomal aneuploidy, with a view to elucidating their potential role in the modulation of vascular endothelial growth factor (VEGF) and nitric oxide (NO). Compared to euploid cells, reduced A_1 and A_{2B} expression was revealed in TR21 CV and MCs. The non-selective adenosine agonist 5′-N-ethylcarboxamidoadenosine (NECA) increased NO, by activating, predominantly, A_1AR and A_{2A}AR through a molecular pathway involving hypoxia-inducible-factor-1 (HIF-1α), and increased VEGF, mainly through A_{2B}. In conclusion the adenosine transduction cascade appears to be disturbed in TR21 through reduced expression of A_{2B} and A_1ARs. These anomalies may be implicated in complications such as fetal growth restriction, malformation and/or SA, well known features of aneuploid pregnancies. Therefore A_1 and A_{2B}ARs could be potential biomarkers able to provide an early indication of SA risk and their stimulation may turn out to improve fetoplacental perfusion by increasing NO and VEGF.

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1. Introduction

15% of human pregnancies are known to end in spontaneous abortion (SA) before 12 weeks of gestation, and immunity, angiogenesis and apoptosis-related genes have all been implicated. In aneuploidy, however, the reported percentage of SA is much higher [1]. One possible reason could be that the chromosomal abnormality itself leads to miscarriage, but if this is the case, the pathogenic mechanism is still unknown.

It has also been suggested that the causes of SA in aneuploidy are no different to those in euploidy, with the increased frequency in the former perhaps being ascribable to a genetically-determined imbalance in the mediators of placental perfusion and uterine contraction [1]. In this scenario, mediators such as endothelial growth factor (VEGF) and nitric oxide (NO) may be involved; indeed, a critical role has been reported for both in placental angiogenesis [2,3]. During gestation, angiogenesis occurs extensively in the placenta and villi to supply the fetus with oxygen and nutrition. This vascular development during embryonic and fetal growth in utero is triggered by hypoxia, a condition that is also known to increase the levels of adenosine (Ado) [4]. This important hormone is locally released from metabolically active cells, or generated extracellularly by the degradation of ATP. Acting through its receptor (AR) subtypes A_1, A_{2A}, A_{2B} and A_3, this nucleoside has been shown to regulate a wide variety of physiological processes, including angiogenesis in hypoxic tissues [5]. In particular, Ado plays an important role in the regulation of VEGF from placental villi in hypoxic conditions, and it also increases NO

synthesis in the fetoplacental endothelium; it is therefore considered to be a major factor in maintaining normal fetoplacental function [6,7].

Although AR expression has been characterized in human placenta from normal pregnancies, no data are as yet available concerning expression and signaling cascades triggered by ARs in aneuploidies [8,9]. Nevertheless, as previously mentioned, aneuploidies very often end in SA, making them a good experimental model for potentially shedding light on the mechanism regulating this event. The aim of this study was therefore to investigate the expression of ARs in first-trimester chorionic villi

(CV) and isolated mesenchymal cells (MC) from both euploid (E) and trisomy (TR) 21 pregnancies, one of the most frequent autosomal aneuploidy; viable cells, namely those obtained via routine chorionic villus sampling, rather than spontaneous abortus tissue, were chosen, as any alteration of ARs in the latter could be a consequence rather than the cause of miscarriage. The rationale behind the study was that elucidating the role of Ado in the modulation of important proangiogenic molecules like VEGF and NO in aneuploid pregnancies may also shed light on the proteins and pathways involved in SA in euploid pregnancy.
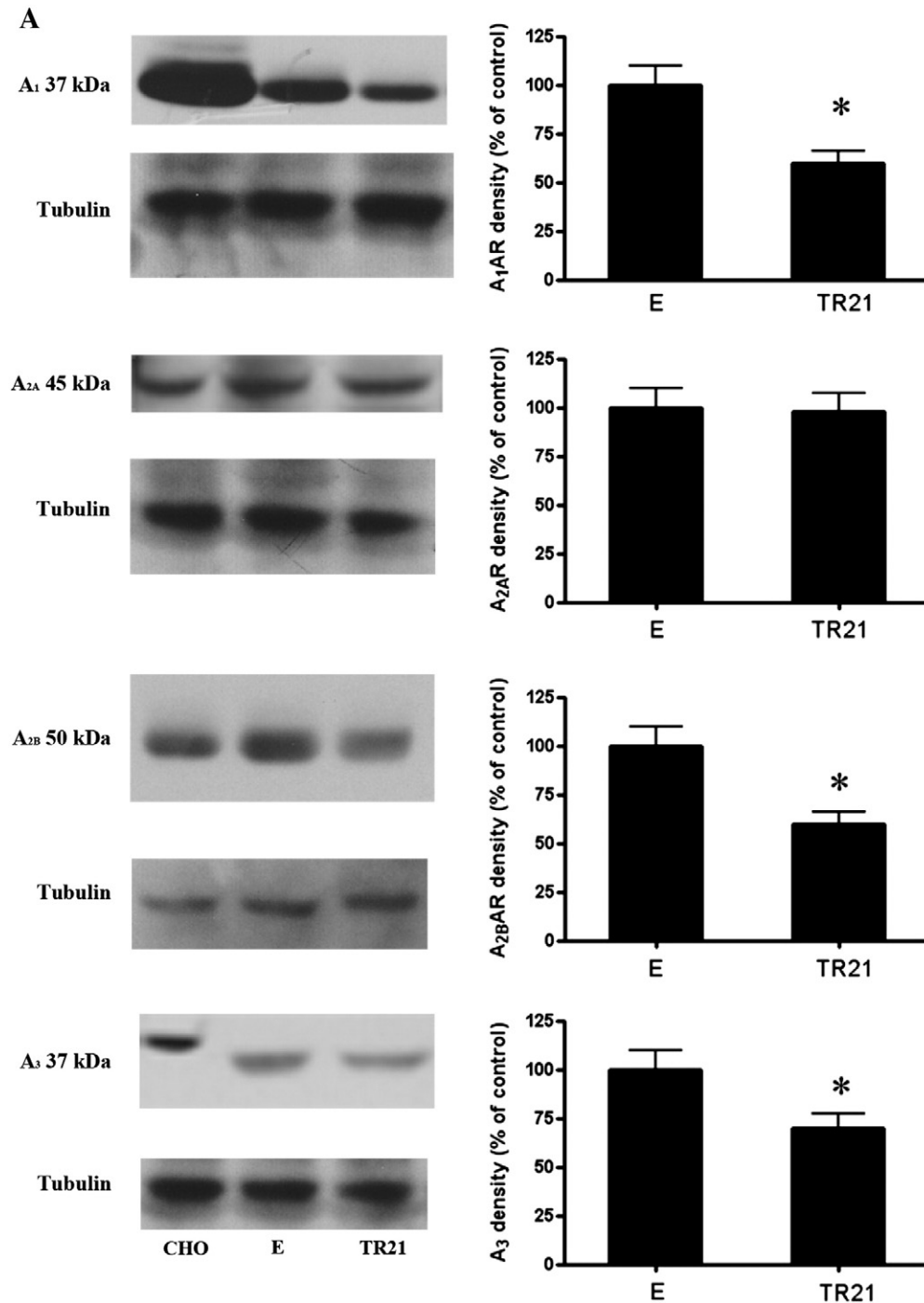
Fig. 1. Expression levels of A_1, A_{2A}, A_{2B}, A_3AR proteins in E and TR21 CV. Representative Western blot analyses of ARs in CV biopsies from women with E (line 2) and TR (line 3) pregnancies at 12 weeks of gestation. CHO cells transfected with the different ARs were loaded as positive control (line 1). Histograms represent % decrease with respect to E pregnancies. Densitometric quantification of Western blots is the mean ± SE values (N = 4 for each group) *P<0.01 vs E CV. (A); Saturation curves of [³H]DPCPX, [³H]ZM 241385, [³H] MRE 2029 F20 and [³H]MRE 3008 F20 binding to A_1, A_{2A}, A_{2B}, A_3ARs in CV biopsies from E (N = 13) and TR21 pregnancies (N = 10) (B).Specific (■) and nonspecific equilibrium binding (▲) were determined as described in the methods. Each value represents the mean ± SEM of experiments performed in duplicate.

B

Fig. 1 (*continued*).

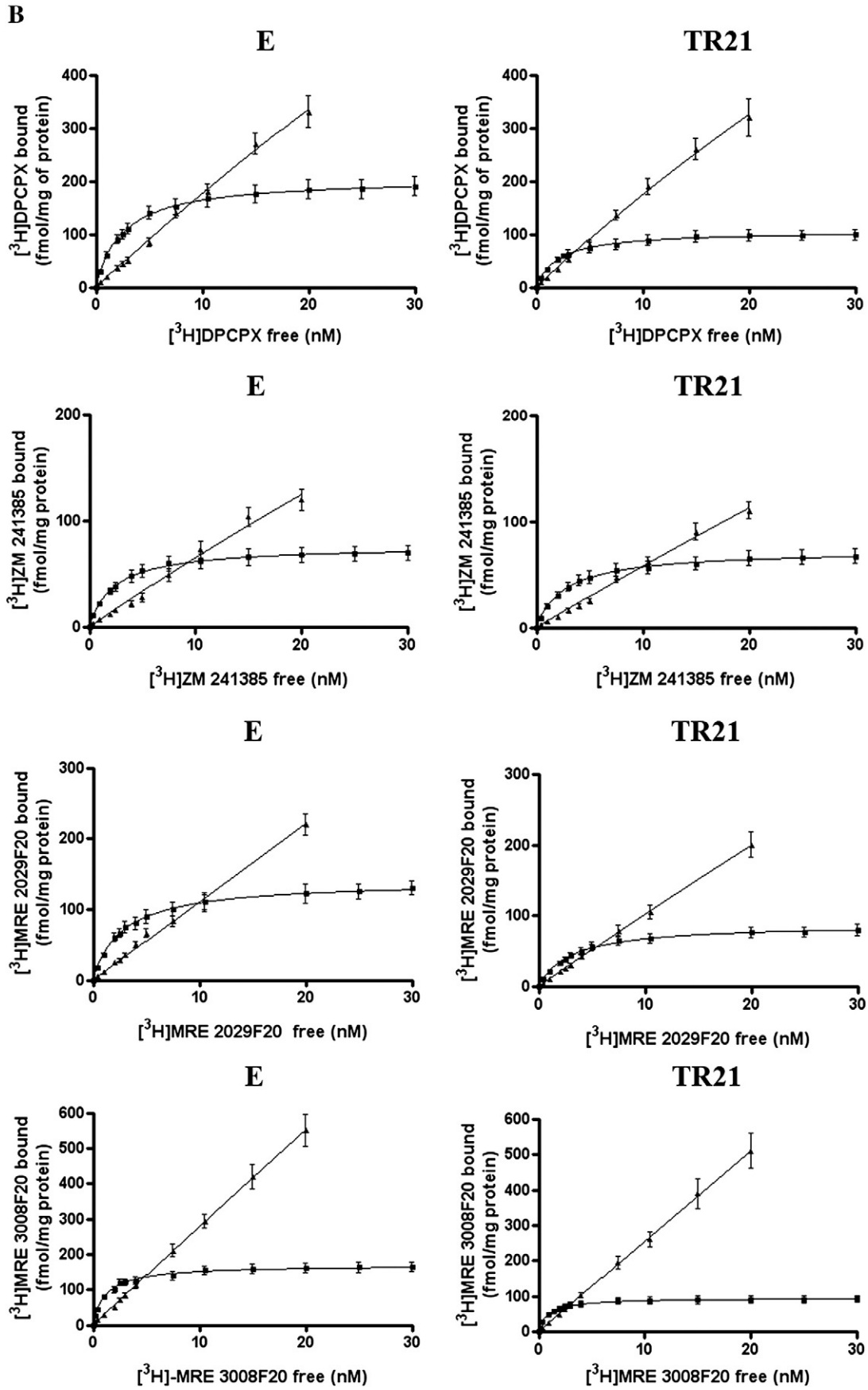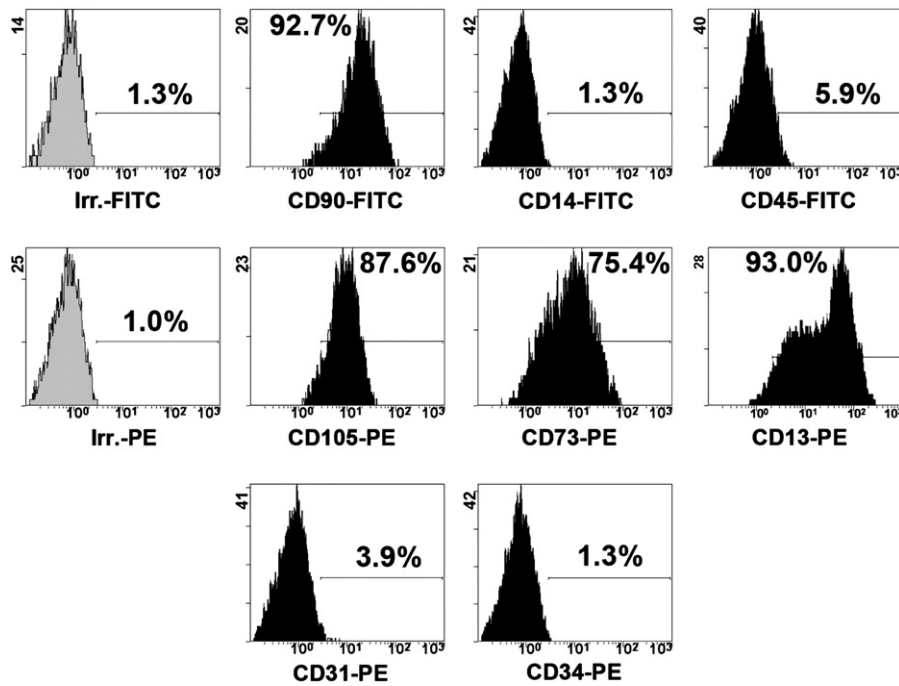**Fig. 2.** Immunophenotyping by flow cytometry analysis of purified CVMCs. Cell surface expression of MSC (CD13, CD73, CD90 and CD105), myeloid (CD14, CD45) and endothelial (CD31, CD34) markers is reported. The number of positive cell is reported in each diagram as % of total gated cells (> 10,000 events were analyzed). As negative control, cells were also stained with isotype-matched irrelevant antibody fluorescence (Irr.-FITC and Irr.PE). A representative sample is reported (N = 3).

2. Materials and methods

2.1. Materials

[^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine ([^{3}H]DPCPX) (specific activity 120 Ci/mmol), was purchased by NEN Research Products (Boston, MA). [^{3}H] (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,32][1,3,6]triazinyl-amino]ethyl)-phenol) ([^{3}H]ZM 241385) (specific activity 20 Ci/mmol), was furnished by Tocris (Boston, MA). [^{3}H]N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahyro-1 H-purin-8-yl)-1-methyl-1 H-pyrazol-3-yl-oxy]-acetamide] ([^{3}H]MRE 2029-F20) (specific activity 123 Ci/mmol) and [^{3}H]5-N-(4-methoxyphenylcarbamoyl)-amino-8-propyl-2-(2-furyl)-pyrazolo[4,3e]-1,2,4triazolo[1,5c]pyrimidine ([^{3}H]MRE 3008 F20) (specific activity 67 Ci/mmol), were synthesized at Amersham International (Buckinghamshire, UK). ARs small interfering RNA (siRNA) were from Santa Cruz DBA (Milano, Italy). RNAiFect Transfection Kit was purchased from Qiagen (Milano, Italy). Antibody against A_1 and A_3 ARs were obtained from Calbiochem Inalco (Milano, Italy). Antibody against A_{2A} was from Alpha Diagnostic, Vinci Biochem (Firenze, Italy). Antibody against A_{2B} was from Santa Cruz, Tebu-bio (Milano, Italy). VEGF ELISA kit was purchased from R&D Systems, Space Import–export (Milano, Italy) and NO ELISA kit was furnished by Merck Chemicals (Nottingham, UK). CD45, CD34, CD14, CD73, CD90, CD13, CD31 antibodies were purchased from BD Biosciences (Milano, Italy), CD105 was from Space Import–export Milano (Milano, Italy). The Assays-on- demand™ Gene expression Products Hs00181231_m1, Hs00169123_m1, Hs00386497_m1, Hs00181232_m1, Hs01573922_m1, Hs00173626_m1 for A_1, A_{2A}, A_{2B}, A_3, CD73 and VEGF, respectively were purchased from Applied Biosystems (Monza, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milano, Italy).

2.2. Study subjects and sample collection

From 2008 to 2011 CV were collected from 71 pregnant women consecutively scheduled for sampling at 12 weeks of gestation. Pregnancies were categorized as follows: 41 E and 30 TR21. Informed consent was

obtained in all cases, and the study was approved by the Ferrara S. Anna University Hospital Ethics Committee.

2.3. Membrane preparation from CV

Membrane preparation from 23 CV (13E and 10TR21) was carried out as described previously with minor modifications [10]. Briefly,
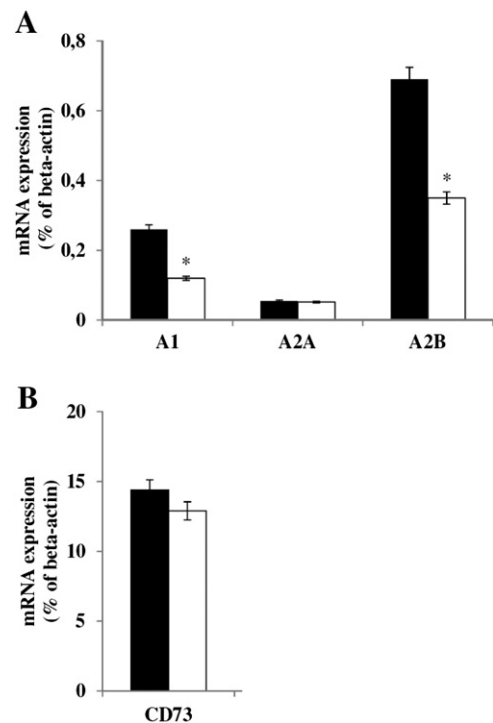

Fig. 3. Expression levels of A_1, A_{2A}, A_{2B} AR and CD73 target genes in E and TR21 CVMCs. Histograms showing the content of AR (A) and CD73 (B) mRNA in E (N = 13, black) and TR21 (N = 8, white) MCs. Data were expressed as percentage of β-actin expression. *P < 0.01, vs. the corresponding receptor in E cells.

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tissue was homogenized in a polytron homogenizer with 50 mM HEPES buffer (pH 7.5) containing 250 mM sucrose, 1 mM EDTA and 2 mM phenylmethyl-sulphonyl fluoride. The homogenate was centrifuged at 600 $\times g$ for 15 min. The pellet was discarded and the supernatant centrifuged at 18,000 $\times g$ for 30 min. The supernatant obtained was further centrifuged at 100,000 $\times g$ for 60 min. The crude membrane pellet was washed twice and finally resuspended in membrane buffer (50 mM HEPES, pH 7.5 containing 4 mM MgCl$_2$).

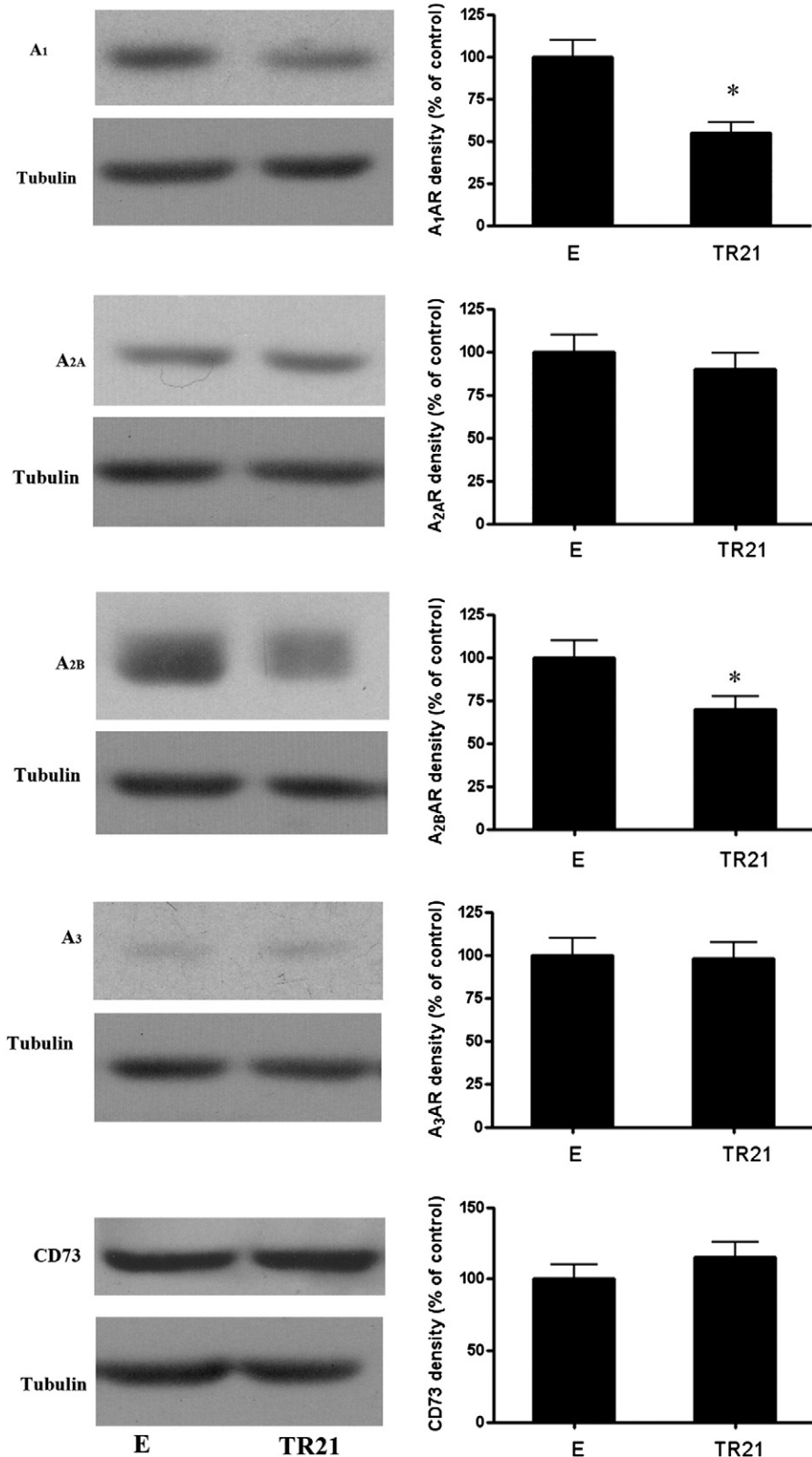**Fig. 4.** Expression levels of A$_1$, A$_{2A}$, A$_{2B}$, A$_3$AR and CD73 proteins in E and TR21 CVMCs. Representative Western blot analyses of ARs and CD73 in MC cells from euploid and TR21 pregnancies at 12 weeks of gestation. Histograms represent % decrease with respect to E pregnancies. Densitometric quantification of Western blots is the mean $\pm$ SE values (N = 3 for each group). *P<0.01 vs E CV.
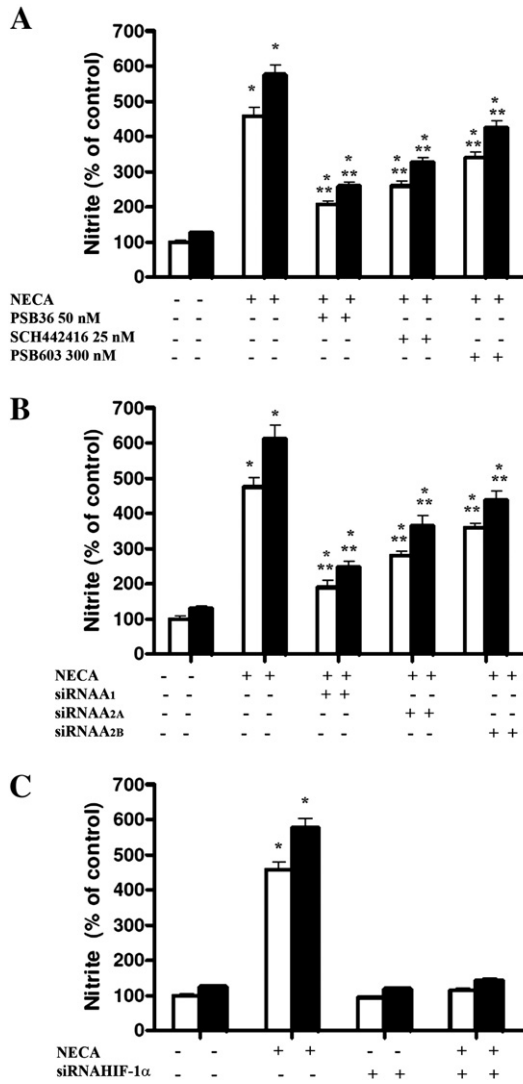
Fig. 5. NECA-stimulated NO secretion in E (white) and TR21 cells (black). NO levels in MC treated with 1 μM NECA in the absence and presence of 50 nM PSB 36, 25 nM SCH 44,216 and 300 nM PSB 603 (A) and with specific siRNAs of A_1, A_{2A}, and A_{2B} ARs (B). Effect of HIF-1α siRNA on NO secretion (C). *P<0.05 vs. the corresponding control (E and A cells without NECA) and **P<0.05 vs. the corresponding NECA (N=5 for each group).

2.4. Binding studies

Saturation experiments of [3H]DPCPX (0.1-30 nM), [3H]ZM 241385 (0.1–30 nM), [3H]MRE 2029 F20 (0.1–30 nM) and [3H]MRE 3008 F20 (0.1–30 nM) to label A_1, A_{2A}, A_{2B} and A_3 ARs, respectively, were carried out in membranes from CV as previously described [11]. 100 μl of membrane homogenate (80 μg of protein assay^{-1}) were incubated in duplicate, in a final volume of 250 μl in test tubes containing 50 mM Tris HCl buffer (10 mM $MgCl_2$ for A_{2A}, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine for A_{2B} and 10 mM $MgCl_2$, 1 mM EDTA for A_3) pH 7.4, with 10–12 different concentrations of each selective radioligand. Non-specific binding was obtained by using PSB 36, SCH 442416, PSB 603 and VUF 5574 1 μM and at the K_D value for each radioligand was 31, 30, 32, 26%, respectively of total binding in euploid and 39, 30, 41, 34%, respectively in trisomy 21 cells.

Bound and free radioactivity were separated, after an incubation time of 120 min at 4 °C, by filtering the assay mixture through Whatman GF/B glass-fiber filters using a cell harvester (Packard Instrument Company).

The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

2.5. Western blot analysis

AR expression was evaluated in CV samples (4E and 4TR21) and in mesenchymal cells (3E and 3TR21) by Western blot. Whole cell lysates, prepared as previously described were resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane [11]. Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for A_1, A_{2A}, A_{2B} and A_3 ARs (1:1000 dilution) in 5% non-fat dry milk in PBS 0.1% Tween-20 overnight at 4–8 °C. Membranes were washed and incubated for 1 hour at room temperature with peroxidase-conjugated species specific secondary antibodies. Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent. Tubulin (1:250) was used to mathematically normalize samples; then signals were expressed as % of control.

2.6. Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad). Mean densitometry data from independent experiments were normalized to the results in control cells. The data were presented as the mean ± S.E.

2.7. MC isolation

MC cells were isolated from a total of 40 CVs (24E,16TR21) as previously described [12]. Only cells taken from back-up cultures were used after karyotype analysis. Cells were maintained in Chang medium D, supplemented with 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37° in 5% CO_2/95% air. All cell treatments with ARs ligands were performed in the presence of adenosine deaminase (ADA).

2.8. Hypoxic treatment

Hypoxic exposure (24 h) was performed in a modular incubator chamber flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2 (MiniGalaxy, RSBiotech).

2.9. Immunophenotyping

MCs isolated from 3 separate CV samples were immunophenotyped. Analysis was performed using an EPICS-XL flow cytometer and EXPO32 software (Beckman Coulter). At least 10,000 events were collected per sample [13].

2.10. Real-time RT-PCR

Quantitative real-time RT-PCR was performed as previously reported [11]. Total cytoplasmic RNA was extracted from MCs (13E, 8TR21) by the acid guanidinium thiocyanate phenol method. For the real-time RT-PCR of A_1, A_{2A}, A_{2B}, A_3 ARs, CD73 and VEGF the assays-on- demand™ Gene expression Products were used. As reference gene the endogenous control human β-actin kits was used, and the probe was fluorescent-labeled with VIC™ (Applied Biosystems, Monza, Italy).

2.11. Immunofluorescence analysis

For HIF-1α detection cells were treated with NECA for 2 h, under hypoxia. MCs (5E, 5TR21) were washed two times with PBS, fixed in 10% paraformaldehyde for 10 min, permeabilized in a PBS solution containing 0.1% of Triton X-100 and incubated for 30 min with PBS plus 5% goat serum and 0.5% bovine serum albumin. The cells were then incubated O.N. at 4 °C in a humidified chamber with anti-HIF-1α

Abs solutions (1:50) containing 0.5% of goat serum and 0.5% of bovine serum albumin in PBS. Excessive antibody was washed away with PBS and rabbit antibodies were detected with fluorescein isothiocyanate- labeled goat anti-rabbit IgG. Coverslips were stained with 4¢,6¢,-diamino-2-phenyl-indole, mounted in DABCO glycerol-PBS and observed on Nikon fluorescent microscope (Eclipse 50i) as previously described [14]. Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Milan, Italy). Levels of hypoxic HIF-1 in euploid cells were the basis for calculation of the additional NECA-mediated increase and for HIF-1 staining in aneuploid cells. The mean intensity of each cell was obtained from the cells pixels that had a higher intensity than that of the mean background intensity. A mean of 150 cells was analyzed for each condition at 40X magnification, at fixed time exposure.

2.12. siRNA treatment of MCs

MCs were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of ARs siRNA, was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit for 72 hours [11]. A non-specific control ribonucleotide sense strand (5'-ACU CUA UCU GCA CGC UGA CdTdT-3') and antisense strand (5'-dTdT UGA GAU AGA CGU GCG ACU G-3') were used under identical conditions.

2.13. Nitrite assay for mesenchymal cells

NOS activity in MCs (5E, 5TR21) was assessed indirectly by measuring nitrite (NO2-) accumulation in the cell culture media using a colorimetric kit (Calbiochem, Milan, Italy). At the end of the treatment period, the nitrite concentration in the conditioned media was determined according to a modified Griess method [14]. Briefly, the NADH-dependent enzyme nitrate reductase was used to convert the nitrate to nitrite prior to quantification of the absorbance, measured at 540 nm by a spectrophotometric microplate reader (Fluoroskan Ascent, Labsystems, Sweden). Sodium nitrite was used as the standard compound.

2.14. Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGF secreted by the MCs (5E, 5TR21) in the medium were determined by ELISA kits. In brief, subconfluent cells (40,000/ml) were seeded in 24-well plates andincubated in the presence of solvent or various concentrations of ado ligands for 24 hours. The medium was collected, centrifuged for 5 min at 900 g to remove floating cells and assayed for VEGF and NO content by ELISA according to the manufacturer's instructions. The data were presented as mean ± SE from four independent experiments.

2.15. Statistical analysis

LIGAND, a weighted nonlinear least-squares curve-fitting program, was used for computer analysis of the data from saturation experiments [11]. Functional experiments were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPAD Prism, San Diego, CA, USA). Data sets were analyzed using Student's t test or analysis of variance (ANOVA) and Dunnett's test (when required). A P-value of less than 0.05 was considered statistically significant. All values in the figures and text are expressed as mean ± standard error (S.E.) of independent experiments and are indicated in the figure legends. Each experiment was performed by using the CV derived from one single donors, and was performed in duplicate (for binding and real-time PCR experiments) or in triplicate (for functional experiments). The experiments were repeated at least three times as indicated from n-values that represent the number of patients used.

3. Results

3.1. AR proteins in E and TR21 CV

Immunoblotting was used to investigate AR expression in CV biopsies from E and TR21 pregnancies. Expression of A_1, A_{2B} and A_3ARs was lower in TR21 CV with respect to E CV, while no difference in A_{2A}AR was observed between the two (Fig. 1A). The specificity of the A_{2B} antibody, recognizing a band of 50 kDa different from the predicted molecular wheight of 36 kDa, was assessed in both untransfected and transfected CHO cells as shown in supplemental Fig. 1. Saturation binding experiments in CV were carried out to evaluate affinity (K_D) and density (Bmax) of ARs. [^{3}H]DPCPX saturation assays revealed A_1ARs with K_D of 2.4 ± 0.3 and 1.9 ± 0.2 nM, and Bmax of 203 ± 21 and 107 ± 12 fmol/mg of protein in E and TR21 samples, respectively; [^{3}H]ZM 241385 saturation studies showed A_{2A}ARs with K_D of 2.5 ± 0.3 and 2.8 ± 0.3 nM, and Bmax of 78 ± 10 and 72 ± 9 fmol/mg of protein in E and TR21 samples, respectively; [^{3}H]MRE2029F20 saturation experiments detected A_{2B}ARs with K_D of 2.8 ± 0.3 and 3.2 ± 0.3 nM, and Bmax of 140 ± 15, and 90 ± 12 fmol/mg of protein in E and TR21 samples, respectively; [^{3}H]MRE3008F20 saturation assays revealed A_3ARs with K_D of 1.2 ± 0.2 and 0.9 ± 0.1 nM, and Bmax of 168 ± 20 and 95 ± 10 fmol/mg of protein in E and TR21 samples, respectively (Fig. 1B). Scatchard plot analysis revealed the presence of an high affinity binding site for each radioligand as suggested by the linearity of the lines. Computer analysis of the data failed to show a significantly better fit to a two site than to a one site binding model, suggesting that under our experimental conditions, there was, primarily, a single class of high affinity binding sites.

3.2. Cell morphology

Primary cultures obtained from CV exhibited a population of embryonal MCs, as revealed by positive immunostaining towards CD13, CD73, CD90 and CD105, while hematopoietic and endothelial cell markers were undetectable (CD14, CD31, CD34, CD45). Flow cytometry demonstrated culture contamination by non-MC cells of less than 5% (Fig. 2).

3.3. AR mRNAs in CVMCs from E and A pregnancies

AR mRNAs were investigated in CVMCs from E and TR21 pregnancies. The order of expression detected in both was $A_{2B} > A_1 > A_{2A}$AR. A_3AR was revealed after 37–38 PCR cycles, suggesting low levels of expression. A_1ARs were found to be downregulated in MCs of TR 21, (0.51 ± 0.05) in comparison to euploid MCs; A_{2B}AR was reduced in TR 21 (0.52 ± 0.05); as for A_{2A}ARs, they were expressed at similar levels to E in TR 21 (0.95 ± 0.1) (Fig. 3A). CD73 expression was similar in E and A cells (0.90 ± 0.1) (Fig. 3B).

3.4. AR proteins in E and A MCs

Western blot analysis was used to quantify ARs in MCs from CV of E and TR21 pregnancies. A_1 and A_{2B} ARs were found to be downregulated in TR21 MCs with respect to E MCs. A_{2A}ARs and CD73 were not significantly altered in TR21. A_3ARs were expressed at low levels in both E and A cells (Fig. 4).

3.5. ARs increase NO secretion in MCs

We evaluated NO production by MCs after treatment with the non-selective agonist 5'-N-Ethylcarboxamidoadenosine (NECA) under hypoxic conditions (24 h). NECA raised NO levels by 459 ± 50% and 466 ± 48% in E and TR21 cells, respectively. Basal NO levels were slightly higher in TR21 than in E cells (39 ± 4 and 49 ± 5 μM in E and TR 21 cells, respectively) (Fig. 5A). The NECA effect was strongly reduced by

1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1 H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1 H-purine-2,6-dione (PSB 36) and 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7 H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 442416), selective antagonists of A_1 and A_{2A} ARs, respectively, suggesting the involvement of A_1 and A_{2A} subtypes, and reduced to a lesser extent by 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB 603), selective antagonist of A_{2B} (Fig. 5A). The effect of AR siRNAs was also tested. A_1AR siRNA was the most potent at reducing NECA-induced stimulation of NO levels, followed by A_{2A} and A_{2B}AR siRNAs (Fig. 5B). siRNA of HIF-1α greatly reduced NECA-stimulated NO increase, suggesting that ARs were acting through HIF-1α modulation (Fig. 5C). After 48 and 72 h posttransfection with siRNA targeting each AR and HIF-1, protein levels were significantly reduced

(Fig. 6A); the specificity of a given siRNA to the other AR subtypes is also shown in Fig. 6B.

We therefore evaluated HIF-1α accumulation after incubation of E and TR21 MCs with NECA under hypoxia (2 h). NECA stimulated HIF-1α accumulation in both cell types, confirming the involvement of this transcription factor (Fig. 7).

3.6. ARs stimulate VEGF secretion in MCs

We tested VEGF production by MCs after treatment with NECA under hypoxia (24 h). NECA increased VEGF levels in a dose-dependent fashion by $314 \pm 32\%$ and $330 \pm 35\%$ in E and A cells, respectively. TR21 cells presented a lower basal level of VEGF (222 ± 26 pg/ml in TR21 vs 404 ± 52 pg/ml in E cells), and NECA showed a lower affinity for
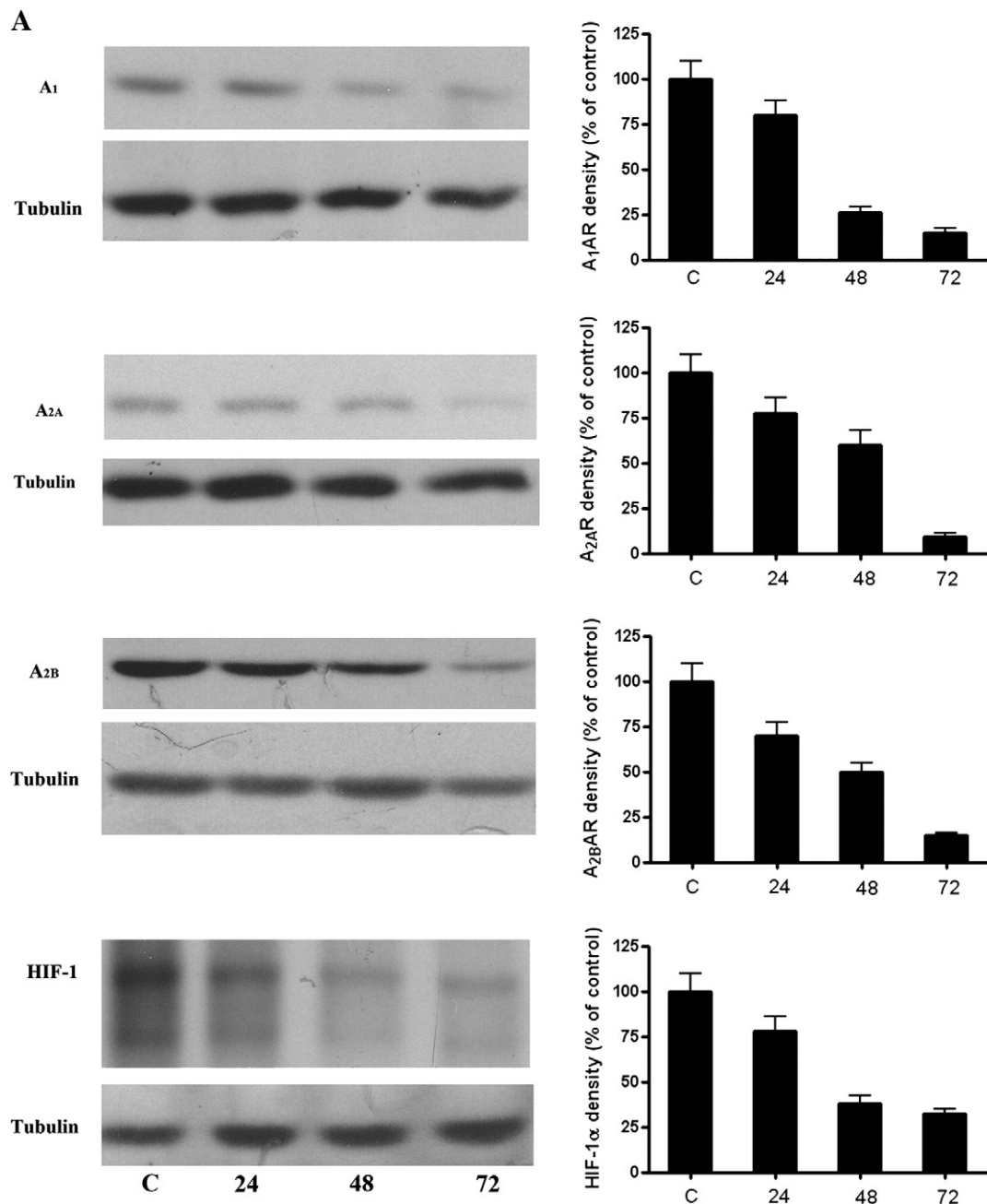

Fig. 6. ARs and HIF-1α silencing by siRNA transfection in MC. Western blot analysis using anti A_1, A_{2A}, A_{2B} and HIF-1 polyclonal antibodies of protein extracts from MC treated with siRNA and cultured for 24, 48 and 72 h (A). Specificity of adenosine receptors siRNAs (B). Western blot analysis using anti A_1, A_{2A}, and A_{2B} receptor polyclonal antibodies of protein extracts from MC transfected with control ribonucleotides (ctr.) or with siRNA of each AR subtype and cultured for 72 h. Tubulin shows equal loading protein.
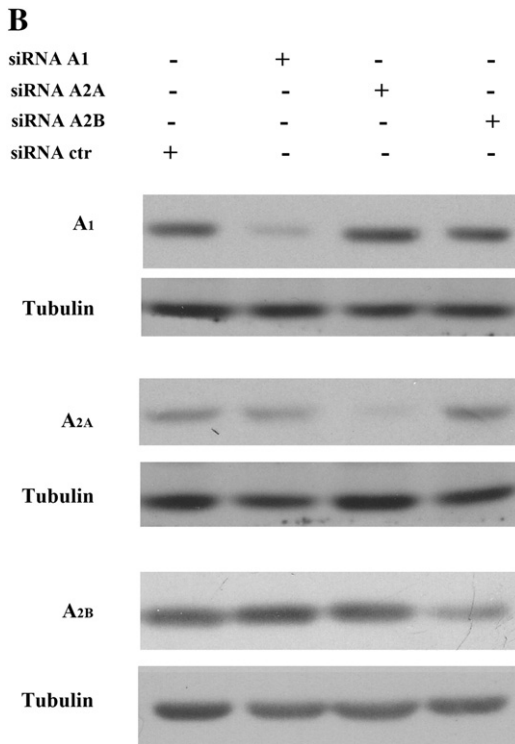
B

Fig. 6 (continued).

stimulation of VEGF secretion in TR21 with respect to E cells (EC$_{50}$ 245 ± 26, 480 ± 50 nM in E and TR21, respectively). The NECA effect was strongly reduced by PSB603 (300 nM), suggesting the involvement of

A$_{2B}$, and to a lesser extent by the A$_{2A}$ antagonist SCH442416 (25 nM), but not by PSB36 (50 nM) the A$_1$ antagonist (Fig. 8A). The effect of AR siRNAs was also tested. A$_{2B}$AR siRNA produced the most potent reduction of NECA-induced stimulation of VEGF, followed by A$_{2A}$AR siRNA, while A$_1$ siRNA has no effect (Fig. 8B). Treatment with nitric oxide synthase antagonist L-N^G-Nitroarginine methyl ester (L-NAME 150 μM) did not reduce NECA-stimulated increase in VEGF, suggesting that ARs were not acting through NO production (data not shown). The effect of NECA on VEGF was also observed on mRNA (2.6 ± 0.2-fold increase) (Fig. 8C).

4. Discussion

Human reproduction is a complex process prone to failure, and several mechanisms, including angiogenesis, inflammatory and immune-related processes, have been considered as possible mediators of SA. Recently, it has been reported that aberrant maternal inflammation associated with SA is closely linked to deficient placental perfusion [15]. Emerging evidence also suggests that Ado, a proangiogenic nucleoside and a sensor of overactive immunity and inflammation, may be involved in determining pregnancy outcome [16–18].

In order to elucidate which ARs may affect the success of this event, we evaluated their expression in TR21, chromosomal abnormalities that very often end in abortion and may therefore represent a good model for elucidating the mechanism regulating miscarriage.

Our findings show, for the first time, a reduction in A$_1$, A$_{2B}$ and A$_3$ARs in CV biopsies obtained from TR21, in comparison to those from euploid pregnancies as evaluated by both western blotting and saturation binding experiments. The A$_{2B}$ antibody reveals a band of 50 kDa, substantially higher than theoretical molecular weight of this receptor subtype. As it has been previously demonstrated that 50–55 kDa immunoreactivity detected in many tissues may not represent the A$_{2B}$AR we evaluated its

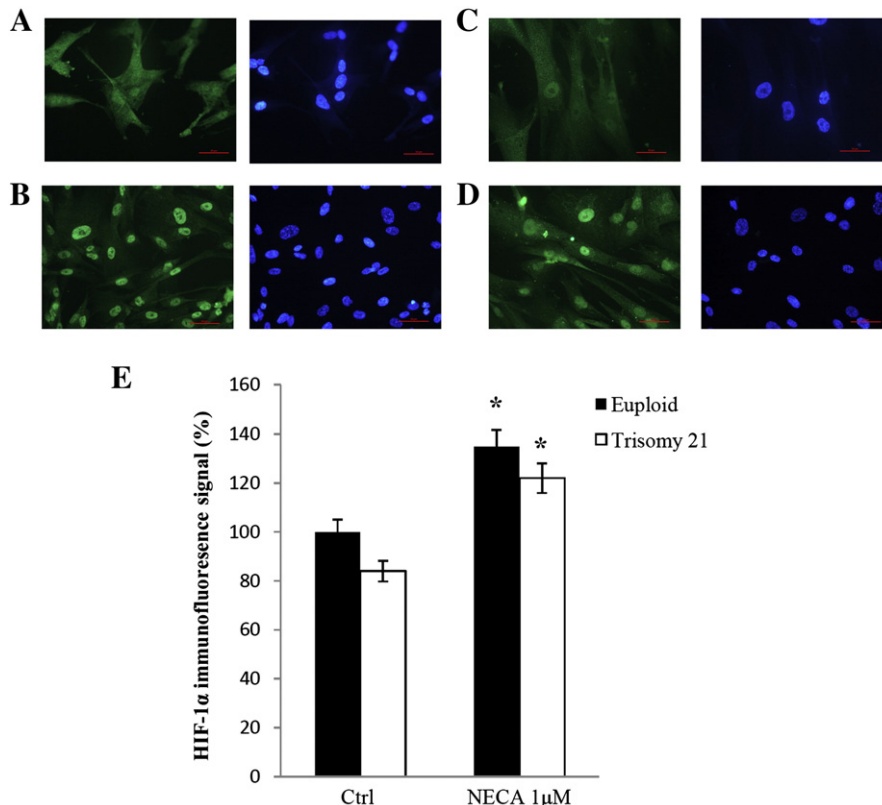**Fig. 7.** Effect of NECA on HIF-1α accumulation in E and TR21 cells. Stimulation of HIF-1α accumulation by NECA (B,D) in CVMC E (A,B) and TR21 (C,D) by means of immunofluorescence analysis; bar graph data (E), expressed as mean ± SE percentage of total HIF-1α staining (N = 3 for each group); *P<0.05 vs. the corresponding control (E and A cells without NECA). Figure shows 1 representative experiment.
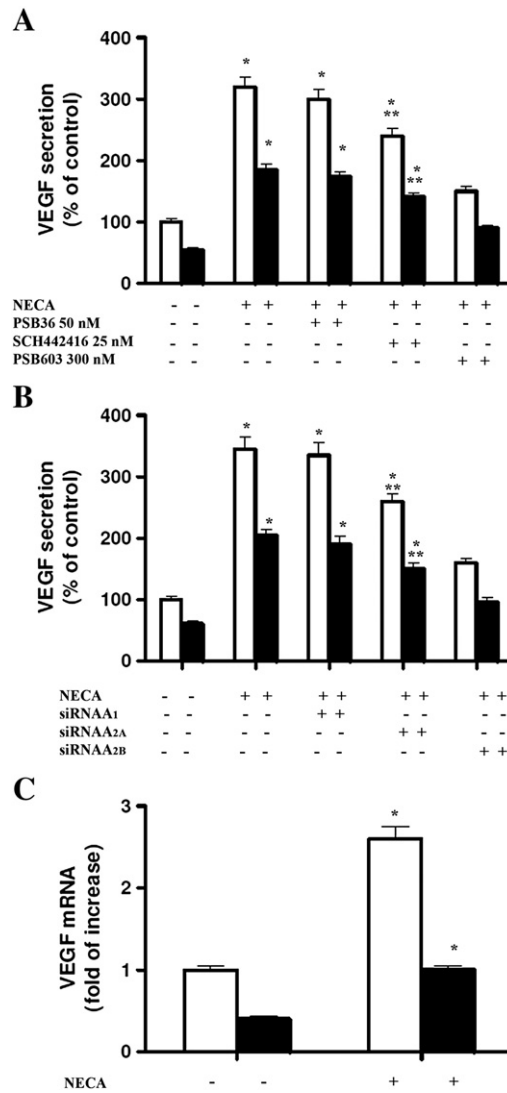
Fig. 8. NECA-stimulated VEGF secretion in E (white) and TR21 cells (black). VEGF levels in CV-derived MCs treated with 1 μM NECA in the absence and in the presence of 50 nM PSB 36, 25 nM SCH 44216 and 300 nM PSB 603 (A), and with specific siRNAs of A_1, A_{2A}, and A_{2B} ARs (B). Induction of VEGF mRNA by NECA in healthy (white) and TR21 (black) cells (C); *P<0.05 vs. the corresponding control (E and A cells without NECA) and **P<0.05 vs. the corresponding NECA (N = 5 for each group).

specificity in transfected and untransfected CHO cells [19]. Our data show that the A_{2B} antibody was specific for A_{2B} receptors, according to literature data [20] and revealed a decrease in receptor density in TR21 CV. In order to quantify the affinity and density of ARs, saturation binding studies were performed. In CV, the adenosine receptor affinities were in the nanomolar range and the receptor densities of A_1, A_{2B} and A_3 subtypes were decreased in TR21 samples. Even though DPCPX and ZM 241385 used as A_1 and A_{2A} radiolabeled ligands can also bind A_{2B} receptors, in the range of concentrations investigated we detected a single class of high affinity binding sites. Similar results have been observed in a recent paper by Varani et al., 2010 in human synoviocytes expressing all four adenosine receptors [21]. We therefore went on to further investigate AR expression in CV MCs, which are routinely withdrawn for genotype analysis. Accordingly, we found, also for the first time, a reduction of A_1 and A_{2B}ARs in A MCs, whilst A_3 was expressed at very low levels in both E and A samples; this suggests that it may be more relevant in other cell types e.g. in preeclampsia, A_3AR expression has been found to be upregulated in trophoblasts, where it regulates MMP-2/9 expression [22]. As a whole, these data support the hypothesis that both A_1 and A_{2B}AR gene products may positively regulate normal pregnancy. Indeed, A_1AR has been reported to play an essential role in protecting the embryo against hypoxia and intrauterine stress [23–25]. As for A_{2B}, it is

known to be involved in increasing angiogenesis through modulation of VEGF, and its genetic loss has recently been found to increase platelet aggregation, suggesting it has a beneficial effect in vascular injury [26–29]. A_{2B}AR is also involved in chorionic vasoconstriction, with pathophysiological implications for preeclampsia (PE) and vascular diseases [30].

Since trisomy 21 placentae are known to feature trophoblastic hypoplasia and hypovascularity, we investigated the potential role played by ARs in NO and VEGF regulation in both TR21 and normal pregnancies [31]; previously, the four ARs had been linked to the angiogenic actions of Ado in endothelial cells, smooth muscle, fibroblasts, monocytes, macrophages, mast and foam cells, all of which are recognized as important sources of proangiogenic factors [7,11,23,32]. We found slightly higher NO content in MCs from TR21 pregnancies than in E cells; A_1 followed by A_{2A}ARs were shown to increase NO production in an HIF-1-dependent fashion, with A_{2B}AR only playing a minor role, confirming previous reports [33,34].

In contrast, A_{2B}AR appeared to be the main subtype involved in VEGF secretion, as indicated by the affinity of NECA and the antagonizing effect mediated by both specific blockers and siRNA of ARs. A_{2A}AR also contributed to VEGF secretion according to literature data [5].

We found reduced VEGF in TR21 with respect to E cells; this was reversed by NECA stimulation, albeit with a lower affinity. These low levels of VEGF production, in addition to the higher NO content, have previously been reported in TR21 stem cells, and may be attributed to the fact that the candidate Down syndrome region gene lies on chromosome 21 and encodes a negative regulator of VEGF–calcineurin signaling [35–37]. Furthermore, NO production, the main vasodilator in pregnancy, has been suggested as a compensatory response for restoring proangiogenic conditions in hypoxic MCs.

4.1. Concluding remarks

As a whole, our data show that the Ado transduction cascade is disturbed in TR21 by two major anomalies, namely downregulation and reduced expression of A_{2B} and A_1ARs. Such anomalies may negatively affect pregnancy to varying degrees; based on the literature, as well as our results, pregnancy can be interpreted as a vascular phenomenon whose destiny depends, among other factors, on the degree of disruption of each of the two ARs, which may be implicated in a range of complications, including SA, fetal malformation, fetal growth restriction and preeclampsia [1,38,39]. Accordingly, stimulation of ARs, particularly A_1 and A_{2B}, may turn out to improve fetoplacental perfusion by increasing NO and VEGF. Our results also suggest that A_1 and A_{2B}ARs may be useful as biomarkers to provide an early indication of SA risk, and, last but by no means least, lay the foundations for future studies investigating the molecular causes of miscarriage.

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References

[1] F. Vesce, C. Scapoli, G. Giovannini, L. Tralli, G. Gotti, A. Valerio, A. Piffanelli, Cytokine imbalance in pregnancies with fetal chromosomal abnormalities, Hum. Reprod. 17 (2002) 803–808.
[2] R.J. Levine, S.E. Maynard, C. Qian, K.H. Lim, L.J. England, K.F. Yu, E.F. Schisterman, R. Thadhani, B.P. Sachs, F.H. Epstein, B.M. Sibai, V.P. Sukhatme, S.A. Karumanchi, Circulating angiogenic factors and the risk of preeclampsia, N. Engl. J. Med. 350 (2004) 672–683.
[3] P. Carmeliet, V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau, A. Nagy, Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele, Nature 380 (1996) 435–439.
[4] M.C. Simon, B. Keith, The role of oxygen availability in embryonic development and stem cell function, Nat. Rev. Mol. Cell Biol. 9 (2008) 285–296.
[5] B.B. Fredholm, A.P. IJzerman, K.A. Jacobson, J. Linden, C.E. Müller, International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors–an update, Pharmacol. Rev. 63 (2011) 1–34.
[6] C. Escudero, L. Sobrevia, A hypothesis for preeclampsia: adenosine and inducible nitric oxide synthase in human placental microvascular endothelium, Placenta 29 (2008) 469–483.
[7] E. George, K. Cockrell, H. Adair, J.P. Granger, Regulation of sFlt-1 and VEGF secretion by adenosine under hypoxic conditions in rat placental villous explants, Am. J. Physiol. Regul. Integr. Comp. Physiol. 299 (2010) R1629–R1633.
[8] I. Fox, L. Kurpis, Binding characteristics of an adenosine receptor in human placenta, J. Biol. Chem. 258 (1983) 6952–6955.
[9] D. Schocken, M. Schneider, Use of multiple radioligands to characterize adenosine receptors in human placenta, Placenta 7 (1986) 339–348.
[10] R. Masnikosa, A. Nikolić, O. Nedić, Affinity modulation of human placental insulin and insulin-like growth factor receptors by lectins, J. Biochem. 143 (2008) 813–820.
[11] S. Gessi, E. Fogli, V. Sacchetto, S. Merighi, K. Varani, D. Preti, E. Leung, S. Maclennan, P.A. Borea, Adenosine modulates HIF-1α, VEGF, IL-8, and foam cell formation in a human model of hypoxic foam cells, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 90–97.
[12] A. Poloni, V. Rosini, E. Mondini, G. Maurizi, S. Mancini, G. Discepoli, S. Biasio, G. Battaglini, E. Berardinelli, F. Serrani, P. Leoni, Characterization and expansion of mesenchymal progenitor cells from first-trimester chorionic villi of human placenta, Cytotherapy 10 (2008) 690–697.
[13] P. Mirandola, G. Gobbi, E. Masselli, C. Micheloni, D. Di Marcantonio, V. Queirolo, P. Chiodera, T. Meschi, M. Vitale, Protein kinase Cε regulates proliferation and cell sensitivity to TGF-1β of CD4+ T lymphocytes: implications for Hashimoto thyroiditis, J. Immunol. 187 (2011) 4721–4732.
[14] S. Merighi, S. Gessi, K. Varani, C. Simioni, D. Fazzi, P. Mirandola, P.A. Borea, Cannabinoid CB(2) receptor modulates microglial cells stimulated with lypopolysaccharide: role of ERK-1/2 kinase signalling in nitric oxide release, Br. J. Pharmacol. 165 (2012) 1773–1788.
[15] S.J. Renaud, T. Cotechini, J.S. Quirt, S.K. Macdonald-Goodfellow, M. Othman, C.H. Graham, Spontaneous pregnancy loss mediated by abnormal maternal inflammation in rats is linked to deficient uteroplacental perfusion, J. Immunol. 186 (2011) 1799–1808.
[16] S. Hitoglou, V. Zournatzi, D. Gougoustamou, M. Hatzistilianou, J. Tzafettas, Adenosine deaminase activity and its isoenzyme pattern in women with recurrent spontaneous abortions, Gynecol. Obstet. Invest. 58 (2004) 126–129.
[17] M.R. Blackburn, J.A. Wubah, J.L. Chunn, L.F. Thompson, T.B. Knudsen, Transitory expression of the A2B adenosine receptor during implantation chamber development, Dev. Dyn. 216 (1999) 127–136.
[18] U. Perni, D. Sezen, A.M. Bongiovanni, I.M. Linhares, D. Skupski, S.S. Witkin, Endogenous adenosine down-modulates mid-trimester intra-amniotic tumor necrosis factor-α production, Am. J. Reprod. Immunol. 62 (2009) 232–237.
[19] J. Linden, T. Thai, H. Figler, X. Jin, A.S. Robeva, Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells, Mol. Pharmacol. 56 (1999) 705–713.
[20] F. von Versen-Höynck, A. Rajakumar, S.A. Bainbridge, M.J. Gallaher, J.M. Roberts, R.W. Powers, Human placental adenosine receptor expression is elevated in preeclampsia and hypoxia increases expression of the A2A receptor, Placenta 30 (2009) 434–442.
[21] K. Varani, F. Vincenzi, A. Tosi, M. Targa, F.F. Masieri, A. Ongaro, M. De Mattei, L. Massari, P.A. Borea, Expression and functional role of adenosine receptors in regulating inflammatory responses in human synoviocytes, Br. J. Pharmacol. 160 (2010) 101–115.
[22] Y.H. Kim, H.S. Hwang, Y.T. Kim, H.S. Kim, Y.W. Park, Modulation of matrix metalloproteinase secretion by adenosine A3 receptor in preeclamptic villous explants, Reprod. Sci. 15 (2008) 939–949.
[23] C.C. Wendler, S. Amatya, C. McClaskey, S. Ghatpande, B.B. Fredholm, S.A. Rivkees, A1 adenosine receptors play an essential role in protecting the embryo against hypoxia, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 9697–9702.
[24] S.A. Rivkees, C.C. Wendler, Adverse and protective influences of adenosine on the newborn and embryo: implications for preterm white matter injury and embryo protection, Pediatr. Res. 69 (2011) 271–278.
[25] D.L. Buscariollo, G.A. Breuer, C.C. Wendler, S.A. Rivkees, Caffeine Acts via A1 Adenosine Receptors to Disrupt Embryonic Cardiac Function, PLoS One 6 (2011) e28296.
[26] M.B. Grant, R.W. Tarnuzzer, S. Caballero, M.J. Ozeck, M.I. Davis, P.E. Spoerri, I. Feoktistov, I. Biaggioni, J.C. Shryock, L. Belardinelli, Adenosine receptor activation induces vascular endothelial growth factor in human retinal endothelial cells, Circ. Res. 85 (1999) 699–706.
[27] I. Feoktistov, A.E. Goldstein, S. Ryzhov, D. Zeng, L. Belardinelli, T. Voyno-Yasenetskaya, I. Biaggioni, Differential expression of adenosine receptors in human endothelial cells: role of A2B receptors in angiogenic factor regulation, Circ. Res. 90 (2002) 531–538.
[28] D. Yang, H. Chen, M. Koupenova, S.H. Carroll, A. Eliades, J.E. Freedman, P. Toselli, K. Ravid, A new role for the A2b adenosine receptor in regulating platelet function, J. Thromb. Haemost. 8 (2010) 817–827.
[29] D. Yang, M. Koupenova, D.J. McCrann, K.J. Kopeikina, H.M. Kagan, B.M. Schreiber, K. Ravid, The A2b adenosine receptor protects against vascular injury, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 792–796.
[30] M.V. Donoso, R. López, R. Miranda, R. Briones, J.P. Huidobro-Toro, A2B adenosine receptor mediates human chorionic vasoconstriction and signals through arachidonic acid cascade, Am. J. Physiol. Heart Circ. Physiol. 288 (2005) H2439–H2449.
[31] S. Pipitone, C. Garofalo, G. Corsello, M. Mongiovì, M. Piccione, E. Maresi, V. Sperandeo, Abnormalities of the umbilico-portal venous system in Down syndrome: a report of two new patients, Am. J. Med. Genet. 120A (2003) 528–532.
[32] A.N. Clark, R. Youkey, X. Liu, L. Jia, R. Blatt, Y.J. Day, G.W. Sullivan, J. Linden, A.L. Tucker, A1 adenosine receptor activation promotes angiogenesis and release of VEGF from monocytes, Circ. Res. 101 (2007) 1130–1138.
[33] F.O. Lima, G.R. Souza, W.A. Jr Verri, C.A. Parada, S.H. Ferreira, F.Q. Cunha, T.M. Cunha, Direct blockade of inflammatory hypernociception by peripheral A1 adenosine receptors: involvement of the NO/cGMP/PKG/KATP signaling pathway, Pain 151 (2010) 506–515.
[34] G. Vásquez, F. Sanhueza, R. Vásquez, M. González, R. San Martín, P. Casanello, L. Sobrevia, Role of adenosine transport in gestational diabetes-induced L-arginine transport and nitric oxide synthesis in human umbilical vein endothelium, J. Physiol. 560 (2004) 111–122.
[35] A.L. Tranquilli, V. Bezzeccheri, C. Scagnoli, L. Mazzanti, G.G. Garzetti, Amniotic levels of vascular endothelial growth factor and nitric oxide at the second trimester in Down's syndrome, J. Matern-Fetal Neonatal Med. 13 (2003) 28–31.
[36] E. Salvolini, M. Orciani, G. Lucarini, A. Vignini, A.L. Tranquilli, R. Di Primio, VEGF and nitric oxide synthase immunoexpression in Down's syndrome amniotic fluid stem cells, Eur. J. Clin. Invest. 41 (2011) 23–29.
[37] K.H. Baek, A. Zaslavsky, R.C. Lynch, C. Britt, Y. Okada, R.J. Siarey, M.W. Lensch, I.H. Park, S.S. Yoon, T. Minami, J.R. Korenberg, J. Folkman, G.Q. Daley, W.C. Aird, Z. Galdzicki, S. Ryeom, Down's syndrome suppression of tumour growth and the role of the calcineurin inhibitor DSCR1, Nature 459 (2009) 1126–1130.
[38] F. Vesce, C. Scapoli, G. Giovannini, A. Piffanelli, A. Geurts-Moespot, F.C. Sweep, Plasminogen activator system in serum and amniotic fluid of euploid and aneuploid pregnancies, Obstet. Gynecol. 97 (2001) 404–408.
[39] F. Vesce, A. Farina, M. Giorgetti, G. Jorizzo, A. Bianciotto, O. Calabrese, G. Mollica, Increased incidence of preeclampsia in pregnancies complicated by fetal malformation, Gynecol. Obstet. Invest. 44 (1997) 107–111.