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Extracellular ATP activates the PLC/PKC/ERK signaling pathway through the P2Y2 purinergic receptor leading to the induction of early growth response 1 expression and the inhibition of viability in human endometrial stromal cells

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ABSTRACT

ATP is an extracellular signaling molecule that activates specific G protein-coupled P2Y receptors in most cell types to mediate diverse biological effects. ATP has been shown to activate the phospholipase C (PLC)/diacylglycerol/protein kinase C (PKC) pathway in various systems. However, little is known about the signaling events in human endometrial stromal cells (hESCs).

The objective of this study was to examine the presence of the P2Y2 receptor and the effects of exogenous ATP on the intracellular mitogen-activated protein kinases (MAPKs) signaling pathway, immediate early genes expression, and cell viability in hESCs. Western blot analysis, gene array analysis, and MTT assay for cell viability were performed.

The current study demonstrated the existence of the P2Y2 purinergic receptor in hESCs. UTP and ATP activated MAPK in a dose- and time-dependent manner. Suramin (a P2-purinoceptor antagonist), neomycin (a PLC inhibitor), staurosporin (a PKC inhibitor), and PD98059 (a MEK inhibitor) significantly attenuated the ATP-induced activation of MAPK. ATP activated ERK1/2 and induced translocation of activated ERK1/2 to the nucleus. The gene array for 23 genes associated with members of the mitogenic pathway cascade and immediate early genes revealed that the expression of early growth response 1 was increased. In addition, MTT assay revealed an inhibition effect of ATP on cell viability.

ATP activated MAPKs through the P2Y2 purinoceptor/PLC/PKC/ERK signaling pathway and induced translocation of ERK1/2 into the nucleus. Further, ATP induced the expression of early growth response 1 and inhibited cell viability in hESCs.

1. Introduction

The female reproductive system is supplied by blood vessels and innervated by autonomic nerves [1,2]. Adenosine 5′-triphosphate (ATP) is released from platelets or autonomic nerves by exocytosis as a neurotransmitter. Extracellular ATP binds to a G protein-coupled P2Y purinoceptor that activates phospholipase C (PLC) and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-triphosphate, which stimulate protein kinase C (PKC) and cytosolic calcium ($[Ca^{2+}]i$) mobilization, respectively. It is widely accepted that many non-neuronal cells that express purinoceptors are activated by ATP released locally in an autocrine or paracrine manner to mediate diverse biological effects [3,4].

The distribution of the autonomic nerves and blood vessels in the female reproductive system leads us to speculate that the released ATP may play a role in regulating endometrial cells such as signaling transduction, gene expression and cell viability. Our previous data demonstrated that P2Y2 receptor mRNA is expressed in human endometrial stromal cells (hESCs), and that ATP is able to activate ERK1/2, and increase the expression of MMP-2, -3, -10, and -24 in hESCs [5]. The present study was designed to examine the existence of the P2Y2 receptor protein and the effects of exogenous ATP on the details of the intracellular signaling pathway and nuclear translocation of mitogen-activated protein kinases (MAPKs) in hESCs.

MAPKs are a group of serine-threonine kinases that transmit externally derived signals to regulate cell growth, differentiation or apoptosis. Mammalian cells contain three major classes of MAP kinase, the extracellular signal-regulated kinases (MAPK/ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) and p38 kinase. MAPKs mediate their effects through phosphorylation of a variety of membrane, cytoplasmic, nuclear and cytoskeletal substrates. Once activated, MAP kinases may translocate to the

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nucleus and phosphorylate nuclear transcription factors, such as Elk-1, that are involved in regulating proliferation, differentiation, and gene expression [6–8]. The present study was designed to examine the effects of ATP on the ERK1/2 signaling pathway, the nuclear translocation of activated ERK1/2 using Western blots, gene array analysis and cell viability assay in hESCs.

2. Materials and methods

2.1. Reagents and materials

ATP, uridine 5′-triphosphate (UTP), suramin, neomycin, and staurosporin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), penicillin–streptomycin, and fetal bovine serum (FBS) were obtained from GIBCO-BRL (Grand Island, NY, USA). P2Y2 receptor antibody (HRP-conjugated goat anti-rabbit antibody) and peptide control were obtained from Alomone Labs (Jerusalem, Israel). PD98059, a MAPK/ERK1/2 kinase (MEK) inhibitor, was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). PD98059, U0126 (Calbiochem, San Diego, CA, USA) and staurosporin were dissolved in dimethyl sulfoxide (DMSO), as suggested by the manufacturer. Phospho-p44/42 MAPK (Thr202/Tyr204) E10 monoclonal antibody (catalog no. 9106), and p44/42 MAPK polyclonal antibody (catalog no. 9102) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). GEArray, GEArray™ Original Series Human Mitogenic Pathway Gene Array and Human MAP Kinase Signaling Pathways GEArray™ Q Series, were purchased from SuperArray Bioscience Corporation (Frederick, MD, USA) (www.superarray.com).

2.2. Tissue collection and hESCs cultures

Samples of proliferative phase endometrium were obtained from 40 cycling women (ages 27–48 years) undergoing hysterectomy who had no history of malignancy. Patient informed consent was obtained from the patients prior to tissue collection. The use of human endometrium was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of Taipei Medical University Hospital.

Isolation of hESCs was performed following the previously described protocol [5,9]. Briefly, 100 mg endometrium was minced and subjected to 0.1% collagenase and 0.1% hyaluronidase, and was digested in a shaking water bath at 37 °C for 1 h. The cells were pelleted by centrifugation at 800 ×*g* for 10 min at room temperature and resuspended in DMEM. The endometrial cells were collected in a 50 ml tube after being passed through a nylon sieve (100 μm) to remove the large cell mass, and collected cells were passed through a smaller nylon sieve (40 μm) to separate stromal cells from epithelial cells. The cells were cultured in DMEM supplemented with 10% FBS and incubated at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. The purity of the stromal cell cultures was determined by immunostaining for vimentin and cytokeratin.

2.3. Treatments

Human ESCs were incubated in a serum-free medium for 4 h prior to treatment. To examine the dose–response relationship, hESCs were treated with increasing concentrations of ATP or UTP (100 nM, 1 μM, 10 μM, or 100 μM) for 5 min. For time-course experiments, hESCs were treated with 10 μM ATP or UTP for 1, 5, 10, or 20 min.

To determine the intracellular signaling pathway, hESCs were treated with suramin (300 μM), neomycin (10 mM), staurosporin (1 μM), or PD98059 (50 μM) in the presence or absence of 10 μM ATP. Human ESCs were pretreated with suramin for 30 min, neomycin for 15 min, staurosporin for 15 min, and PD98059 for 30 min prior to ATP treatment. The cells were collected 10 min after ATP exposure.

2.4. Western blot analysis

The hESCs were washed with ice-cold PBS and lysed with 100 μL of cell lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1.0 mM PMSF, 10 μg/mL leupeptin, and 100 μg/mL aprotinin) at 4 °C for 30 min. The cell lysate was centrifuged at 10,000 ×*g* for 5 min, and the supernatant was collected for Western blot analysis. The amount of protein was quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Aliquots (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis under a reducing condition [10]. The proteins were electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Biosciences, UK), following the procedures of Towbin et al. [11]. These membranes were probed with a mouse monoclonal antibody directed against the phosphorylated forms of ERK1 and ERK2 (P-MAPK, $p42^{mapk}$ and $p44^{mapk}$, respectively) at 4 °C for 16 h. Alternatively, membranes were probed with a rabbit polyclonal antibody for p42/p44 MAPK which detects total MAPK (T-MAPK) levels. After washing, the membranes were incubated with HRP-conjugated goat anti-mouse secondary antibody, and the signal was visualized using an ECL system (Amersham Biosciences, UK) followed by exposure to X-ray film. The autographs were scanned and quantified with Image Pro Plus software (Media Cybernetics, Inc., Silver Spring, MA, USA).

For the anti-P2Y2 receptor study, these membranes were probed with a rabit polyclonal antibody directed against the P2Y2 receptor at 4 °C for 16 h. Alternatively, membranes were incubated with a mixture of the antibody–antigen (for negative control) in 0.5 ml PBS-1% BSA for 1 h, according to manufacturer's protocol.

2.5. Subcellular fractionation

Cytosolic and nuclear extracts were performed following the protocol reported by Yeh et al. [12]. Human ESCs were treatment in the absence or presence of 10 μM ATP for 10 min. To prepare the cytosolic and nuclear extracts of cells, the hESCs were washed with ice-cold PBS and lysed in 100 μL hypotonic buffer (20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol (DTT), and protease and phosphatase inhibitors) at 4 °C for 30 min. After centrifugation at 4000 ×*g* at 4 °C for 10 min, the supernatant was collected as cytosolic extracts. The pellets of nuclei were resuspended in a high salt buffer (20 mM HEPES (pH 7.4), 0.4 M NaCl, 1 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 mM sodium fluoride) and then incubated on ice for 30 min. The supernatant recovered after centrifugation was used as nuclear extracts.

2.6. Total RNA isolation

Total RNA was isolated using an RNeasy Mini Kit (Qiagen GmbH). Briefly, cells were disrupted in a buffer containing guanidine isothiocyanate. Ethanol was then added to the lysate. The sample was then applied to the RNeasy mini column. Total RNA was bound to the membrane, contaminants were efficiently washed away, and high-quality RNA was eluted in RNase-free water. The RNA concentration was determined based on absorbance at 260 nm.

2.7. Gene array analysis

Human ESCs were treated with 10 μM ATP for 30 min in the absence or presence of PD98059 (pretreated for 30 min before ATP exposure) or 10 μM ATP for 24 h prior to RNA extraction. Five micrograms of extracted total RNA was used as a template for the synthesis of cDNA probes with dNTP mix containing biotin-16-dUTP incorporation using the RT-Labeling Kit (SuperArray, Frederick, MD, USA). Annealing of RNA with primers was performed at 70 °C for 3 min. Samples were cooled to 42 °C, and kept at 42 °C for 2 min before labeling with biotin-16-dUTP. The cDNA probe was denatured by heating at 94 °C for 5 min, and chilling quickly on ice. The GEArray membrane spotted with 23 gene-specific cDNA fragments (or the GEArray™ Q Series membrane spotted with 96 gene-specific cDNA fragments) was wet with deionized H2O, and was prehybridized with GEAhyb hybridizational solution containing heat-denatured sheared salmon sperm DNA at 60 °C for 1 to 2 h. The membrane was incubated with the denatured cDNA probe overnight with continuous agitation at 60 °C, then washed twice with pre-warmed 2XSSC containing 1% SDS for 20 min at 60 °C, and twice with pre-warmed 0.1XSSC containing 0.5% SDS for 20 min at 60 °C. The membrane was blocked with GEAblocking solution incubated with alkaline phosphatase-conjugated streptavidin, washed with washing buffer, incubated with CDP-*Star*, a chemiluminescent substrate, and exposed to X-ray film. Each GEArray membrane was spotted with a negative control for pUC18 DNA as well as two positive control genes, β-actin and GAPDH. The relative abundance of a particular transcript was estimated by comparing its signal intensity to the signal derived from β-actin and GAPDH. The intensity of the array of spots was converted into numerical data using Image Pro Express 4.5 software.

2.8. MTT assay for cell viability

Cell viability was assessed by MTT assay, following the manufacturer's protocol (ATCC 30-1010K). Briefly, the hESCs were seeded into 96-well plates at a cell density of 1×10^4 cells per well and incubated in 100 μL (DMEM+10%FBS). The cells were then treated with increasing concentrations of ATP (0, 0.1, 1, 10, 100 μM) for 24 h. Ten microliters of MTT Reagent was added into each well and incubated for 2 to 4 h until intracellular purple precipitate was visible under an inverted microscope. One hundred microliters of Detergent Reagent was added into each well and left at room temperature in the dark for 3 h. The precipitated intracellular purple formazan was solubilized and absorbance was recorded and quantified at 550 nm by scanning with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

2.9. Statistical analysis

MAPK activity was expressed as a relative ratio of basal levels. Independent replicates of experiments in this study were performed with cells from 3 different patients. Data were represented as means ± standard error (SE). Statistical analysis was performed by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered significant at $p<0.05$.

3. Results

3.1. Expression of the P2Y2 receptor protein level in hESCs

We previously demonstrated the expression of the P2Y2 receptor mRNA in hESCs using RT-PCR [5]. As shown in the present study, a 39 kDa P2Y2 receptor protein was demonstrated using Western blot analysis (Fig. 1).

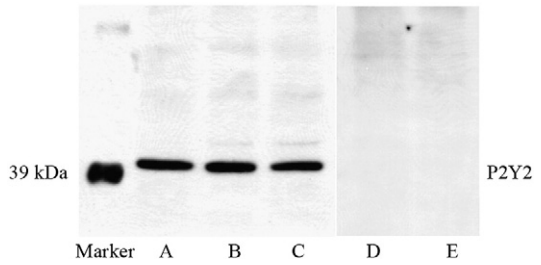

Fig. 1. The P2Y2 purinergic receptor is demonstrated in hESCs obtained from three different patients (A, B, C) using Western blot analysis. The negative control is showed in (D, E), antibody pre-absorbed with antigen.

3.2. Effects of UTP and ATP on MAPK activation

As shown in Fig. 2A, UTP activated MAPK in hESCs in a dose-dependent manner. A significant effect was observed at 1 μM, with a maximum effect noted at 100 μM; there was a statistical difference between the cells treated with 10 μM and those treated with 100 μM of UTP. In time-course experiments, UTP was capable of inducing MAPK activity promptly. A significant effect was seen within 5 min after treatment, and the activation of MAPK was sustained for at least 15 min. As shown in Fig. 2B, the effect of UTP reached maximum at 10 min, and was 5 times greater than the basal level. In addition, as shown in Fig. 2C, ATP activated MAPK in hESCs in a dose-dependent manner. A significant effect was observed at 10 μM with a maximum effect noted at 100 μM. In addition, ATP was capable of rapidly inducing MAPK activity (Fig. 2D). A significant effect was seen within 5 min after treatment, and the activation of MAPK was sustained for at least 15 min. As shown in the study, the effect of ATP reached maximum at 10 min.

3.3. P2-purinergic receptor and ATP-induced MAPK activation

As shown in Fig. 1, the P2Y2 purinergic receptor was expressed in hESCs. Human ESCs were pretreated with 300 μM suramin, a P2 purinoceptor antagonist [13], for 30 min prior to administration of ATP. The co-treatment with suramin and ATP greatly reduced MAPK activity compared to ATP treatment alone (Fig. 3A).

3.4. Involvement of PLC, PKC, and MEK in ATP-induced MAPK activation

As shown in Fig. 3B, treatment of hESCs with neomycin significantly inhibited the ATP-induced activation of MAPK. The combined treatment with neomycin and ATP significantly attenuated MAPK activity by 80%, when compared with ATP treatment alone.

Staurosporin significantly attenuated the ATP-induced activation of MAPK (Fig. 3C). Concomitant treatment with the PKC inhibitor and ATP attenuated MAPK activation by 60%, when compared to the level stimulated by ATP alone.

MEK is the immediate activator of MAPK [14]. The MEK inhibitor, PD98059, significantly decreased the ATP-induced activation of MAPK in hESCs. Simultaneous treatment with PD98059 and ATP reduced

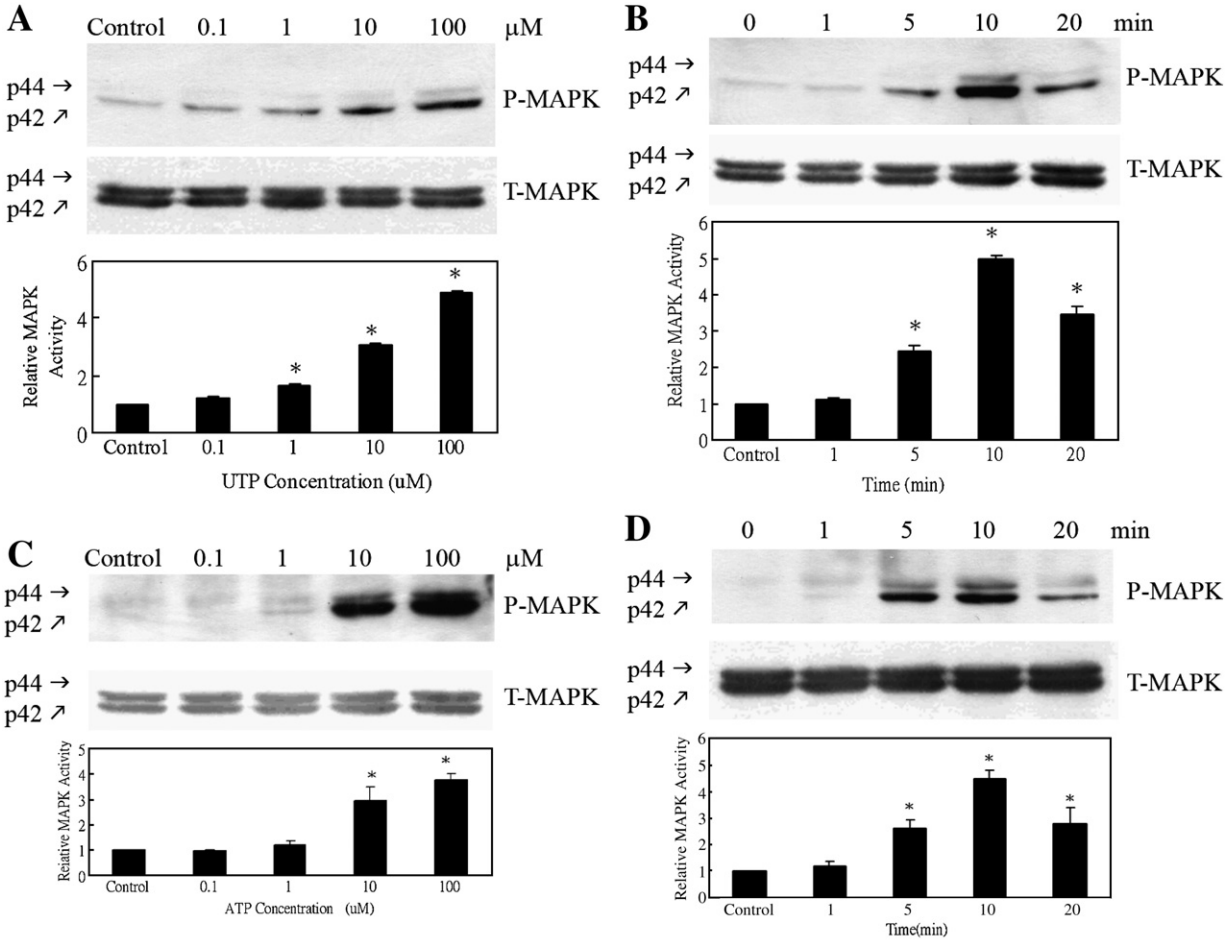

Fig. 2. The dose–response of UTP (A) or ATP (C) on MAPK activation in hESCs. Human ESCs were treated with increasing concentrations of UTP or ATP (0, 100 nM, 1 μM, 10 μM or 100 μM) for 5 min as described in Materials and Methods. The time course of UTP (B) or ATP (D) on MAPK activation in hESCs. Human ESCs were treated with 10 μM UTP or ATP for 0, 1, 5, 10 or 20 min, as described in Materials and Methods. The MAPK were detected by Western blot analysis. The data are shown as the relative ratio to basal levels. Values are presented as the mean±SE of 3 different patients. Differences were considered significant at $p<0.05$, *.

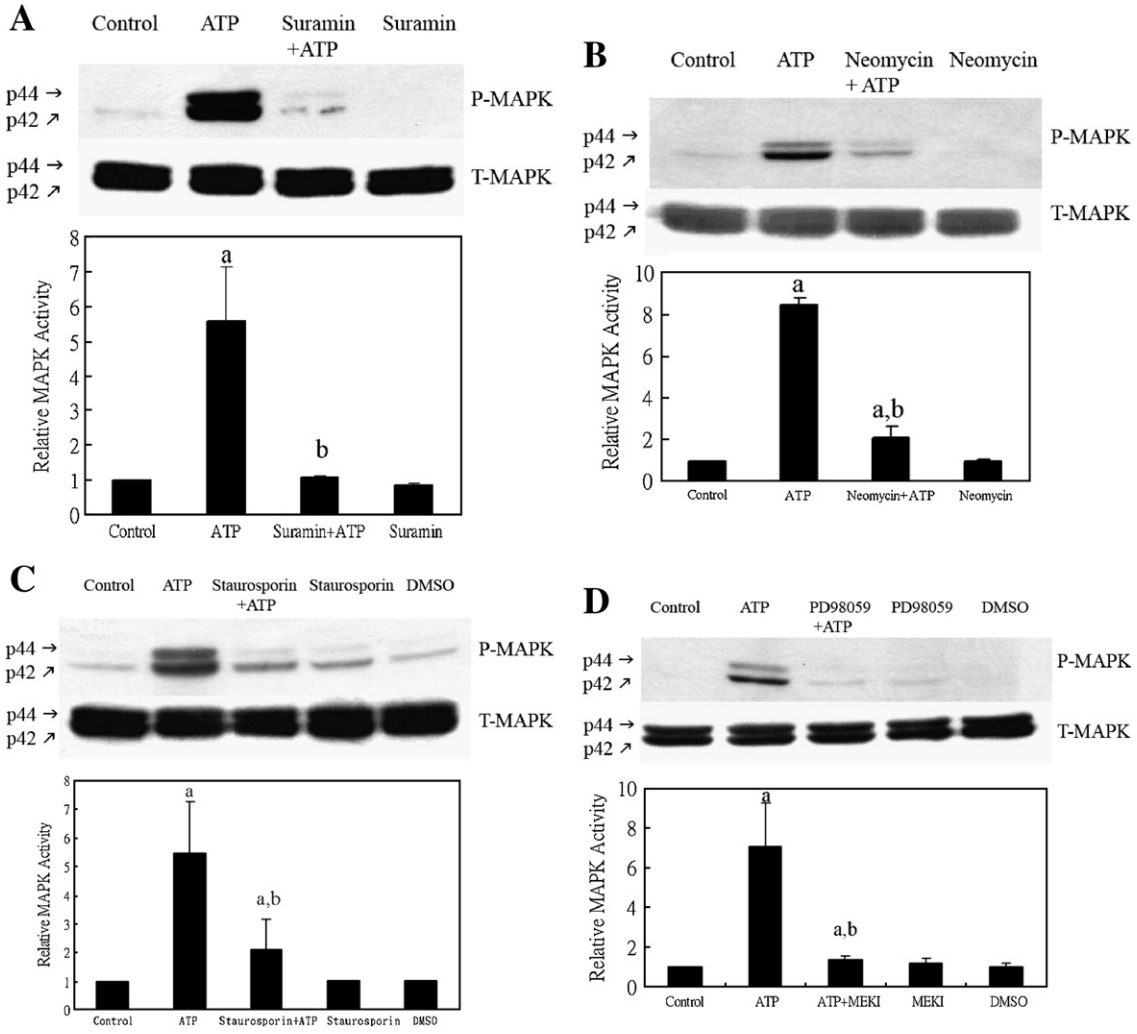

Fig. 3. The effect of suramin (A), neomycin (B), staurosporin (C), or PD98059 (D) on ATP-induced MAPK activation in hESCs. Human ESCs were treated with 10 μM ATP in the presence or absence of suramin (300 μM), neomycin (10 mM), staurosporin (1 μM) or PD98059 (50 μM), as described in Materials and Methods. The MAPK were detected by Western blot analysis. The data are shown as the relative ratio to basal levels. Values are presented as the mean ± SE of 3 different patients. Differences were considered significant at $p<0.05$. a, $p<0.05$ vs. control; b, $p<0.05$ vs. ATP.

MAPK activity to about 80% of the level stimulated by ATP alone (Fig. 3D).

3.5. ATP induces nuclear translocation of ERK1/2

As shown in Fig. 4A, ATP induced translocation of phosphorylated ERK1/2 from the cytosol fraction into the nuclear fraction. Ten μM of ATP significantly increased phosphorylated MAPK activity in the nuclear fraction, when compared with the control group. The effects of ATP on ERK1/2 translocation was completely blocked in the presence of PD98059 or U0126 (Fig. 4B).

3.6. Gene array analysis

Superarray analysis for 23 genes related to members of the mitogenic pathway cascade and immediate early genes revealed that ATP increased the expression of early growth response 1 (egr-1: spots 2E and 2F) (Fig. 5A-I and A-II). These genes include: ATF-2 (creb-2) (spots 1A and 1B), c-*fos* (spots 1C and 1D), c-*jun* (spots 1E and 1F), c-*myc* (spots 2A and 2B), CREB (spots 2C and 2D), egr-1 (spots 2E and 2F), elk-1 (spots 3A and 3B), elk-3 (spots 3C and 3D), ERK1 (spots 3E and 3F), ERK2 (spots 4A and 4B), JNK1 (spots 4C and 4D), JNK2 (spots 4E and 4F), max (spots 5A and 5B), MEK1 (spots 5C and 5D), MEK2 (spots 5E and 5F), MEKK1 (spots 6A and 6B), MEKK3 (spots 6C and 6D), MKK3 (spots 6E and 6F), MKK4 (JNKK1) (spots 7A and 7B), MKK6 (spots 7C and 7D), p38 MAPK (spots 7E and 7F), raf (c-raf-1) (spots 8A and 8B), SRF (serum response factor) (spots 8C and 8D), pUC18 (spots 1G and 2G), and β-actin (spots 3G and 4G). The relative abundance of egr-1 in the ATP-treated group was 5.6-fold of the control group, when normalizing their signal intensities to the signals derived from GAPDH (GAPDH: spots 5G, 6G, 7G, 8E, 8F, and 8G). The data are shown as the relative ratio to GAPDH levels. Values are presented as the mean ± SE of 3 different patients. Differences were considered significant at $p<0.05$, *. As shown in Fig. 5B-II, the effects of ATP on the expression of egr-1 (Fig. 5B-I) was significantly downregulated in the presence of PD98059.

Superarray analysis for 96 genes related to the human MAP kinase signaling pathways revealed that the expression of egr-1 (spots: 1e) was increased in the presence of 10 μM ATP for 30 min (Fig. 5C-II). These genes include: ATF-2 (creb-2) (spots: 3a), c-*fos* (spots: 7e), c-*jun* (spots: 4 f), c-*myc* (spots: 5k), CREB (spots: 3d), egr-1 (spots: 1e), elk-1 (spots: 2e), ERK1 (spots: 8i), ERK2 (spots: 2i), JNK1 (spots: 3j), JNK2 (spots: 5j), max (spots: 8j), MEK1 (spots: 8f), MEK2 (spots: 2 g), MEKK1 (spots: 8 g), MEKK3 (spots: 4 h), MKK3 (spots: 3 g), MKK4 (JNKK1) (spots: 4 g), MKK6 (spots: 6 g), p38 MAPK (spots: 7i), raf (c-raf-1) (spots: 4l), pUC18 (spots: 1 m, 2 m, 3 m), and β-actin (spots: 7n,

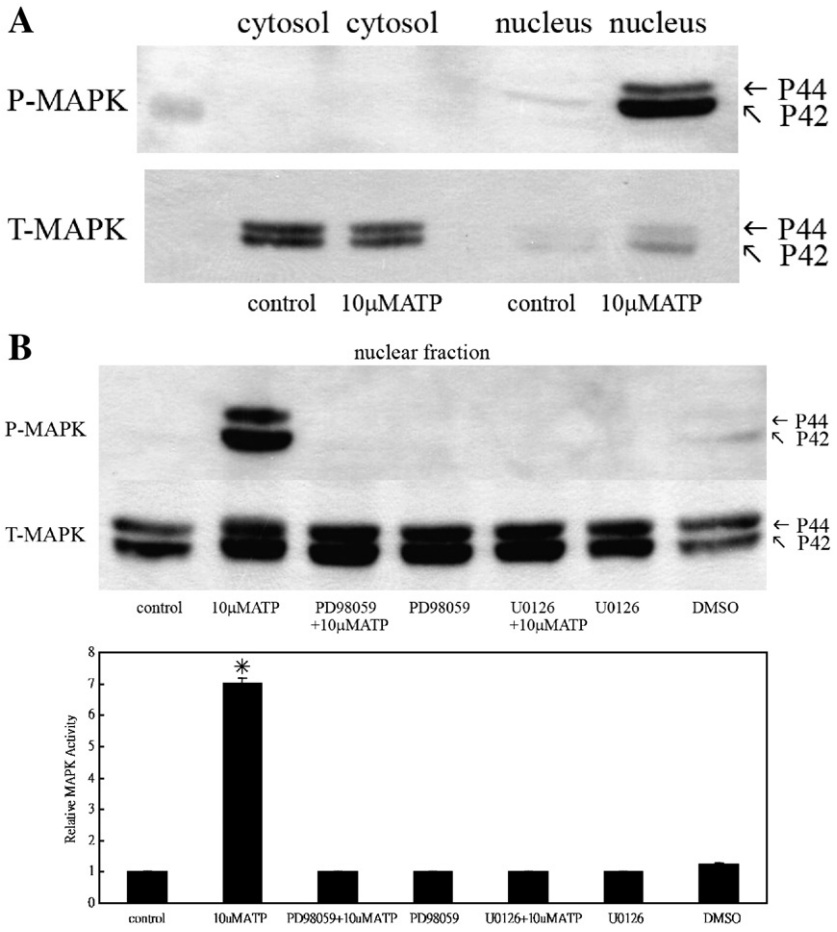

Fig. 4. (A) ATP activated MAPK and induced translocation of activated MAPK from the cytosol to the nucleus. Human ESCs were treated in the absence or presence of 10 μM ATP for 10 min as described in Materials and Methods. (B) The effect of ATP on MAPK activation was attenuated by PD98059 and U0126 in hESCs. The data are shown as the relative ratio to basal levels. Values are presented as the mean±SE of 3 different patients. Differences were considered significant at $p<0.05$, *.

8n). The relative abundance of egr-1 in the ATP-treated group was 4.6-fold of the control group, when normalizing their signal intensities to the signals derived from GAPDH (spots: 7 m, 8 m). The data are shown as the relative ratio to GAPDH levels. Values are presented as the mean±SE of 3 different patients. Differences were considered significant at $p<0.05$, *. As shown in Fig. 5C-III, the effects of ATP on the expression of egr-1 was significantly downregulated in the presence of 10 μM ATP for 24 h.

3.7. The effects of ATP on hESC proliferation

Fig. 6A demonstrates the relationship between cell number and absorbance (OD) for hESCs over a range of $(0.1\text{-}2)\times10^4$ cells per well in a 100 μL culture medium. Excellent linearity could be observed for the hESCs tested up to 2×10^4 cells per well. As shown in Fig. 6B, ATP inhibited cell viability in hESCs in a dose-dependent manner. A significant effect was observed at 10 μM when compared with the control group. The maximum inhibitory effect of 54% was noted at 100 μM ATP. The effects of ATP (10 μM and 100 μM) on cell viability were completely blocked in the presence of PD98059 (50 μM) or U0126 (50 μM) (Fig. 6C and D).

4. Discussion

The present study demonstrated that the P2Y2 purinergic receptor protein was expressed in hESCs, and ATP was able to activate the ERK1/2 through the P2 purinergic receptor, PLC, PKC and MEK signaling pathway. In addition, ATP-activated MAPKs increased the expression of egr-1 in hESCs, and ATP inhibited hESC proliferation.

Purinergic receptors have been identified as P1 and P2 receptors [15]. P2 receptors for extracellular nucleotides are divided into the ion channel receptors (P2X) and the G-protein-coupled receptors (P2Y). Currently, eight subtypes of the P2Y family have been cloned and functionally characterized [4]. For the P2Y receptors, intracellular signaling cascades are the main routes of communication between G-protein-coupled receptors and regulatory targets in the cell. The P2Y2 receptor, which was cloned from several mammalian species, has an equal or higher response to UTP than to ATP [16–18]. Previously, we demonstrated that P2Y2 mRNA was expressed in hESCs [5]. The demonstration of the P2Y2 purinergic receptor in hESCs implies that ATP may play a role in regulating endometrial function.

The expression and activation of MAP kinase was demonstrated during the menstrual cycle in human endometrium [19]. The MAPK family represents an effector for several stimuli imparted by hormones, growth factors, and mechanical and environmental stresses [6]. MAPK/ERK1 and 2 are phosphorylated and activated by MEKs [20,21]. It is reported that 8-Br-cAMP activated PLD through PKA and ERK1/2 in hESCs [22]. In addition, gonadotropin-releasing hormone and TGF-β signaling through MAPK/ERK resulted in differential regulation of fibronectin expression in endometrial cells [23]. Our results demonstrated that ATP is capable of activating ERK1/2 in hESCs through the signaling cascade of P2-purinoceptors, PLC, PKC and MEK. The current study further examined the intracellular functions of activated ERKs in hESC.

Extracellular ATP binds to a purinergic receptor. ERKs play an important role in relaying the signals from receptors on the cell membrane to cytoplasmic targets and nuclear targets including

transcription factors or early response genes. In the nucleus, activated ERK1/2 continuously phosphorylates Elk-1, leading to the nuclear accumulation of transcription factors, such as c-*fos*, which is responsible for DNA synthesis [24]. In the present study, we examined the effect of ATP on 23 genes of members of the mitogenic pathway cascade and immediate early genes. These genes include: ATF-2 (creb-2), c-*fos*, c-*jun*, c-*myc*, CREB, egr-1, elk-1, elk-3, ERK1, ERK2, JNK1, JNK2, max, MEK1, MEK2, MEKK1, MEKK3, MKK3, MKK4 (JNKK1), MKK6, p38 MAPK, raf (c-raf-1), and SRF (serum response factor). Among these genes, only the expression of egr-1 was elevated significantly (Fig. 5A), and the effect of ATP on egr-1 expression was meaningfully attenuated by the MEK inhibitor, PD98059 (Fig. 5B).

Early growth response factor-1 (egr-1) is an immediate early gene which is rapidly activated in quiescent cells by mitogens. Egr-1 is known to be involved in many processes related to cell growth, differentiation and injury repair [25,26]. Extracellular ATP upregulated the expression of egr-1, egr-2, and egr-3 in cultured rat primary cortical neurons [27]. In addition, through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor, extracellular ATP plays an autocrine/paracrine regulator role in fibroblast growth [28]. In the reproductive system, FSH and LH have been shown to induce the expression of the Egr-1 gene, which may mediate molecular programs of proliferation and/or differentiation during follicle growth, ovulation, and luteinization [29]. Our results revealed that extracellular ATP plays an important part in inducing the expression of egr-1 via the ERK signaling pathway in hESCs.

Previously, we reported the effects of exogenous ATP on MAPKs and gene expression in human granulosa-luteal cells (hGLCs) [30,31]. When compared with the current study, we found that ATP exerts different responses in various cells of the female reproductive system. In hGLCs, a significant effect of ATP on ERK1/2 activation was observed at 1 μM, with a maximum effect seen at 10 μM, and there was no statistical difference between cells treated with 10 μM and 100 μM ATP. ATP was capable of rapidly inducing ERK1/2 activity. A significant effect was seen within 5 min after treatment, and the maximal effect was observed at the 5-min time-point in hGLCs. In contrast, no effect of ATP on ERK1/2 activation in the hESCs was noted at 1 μM. A significant influence of ATP on ERK1/2 activation was observed at

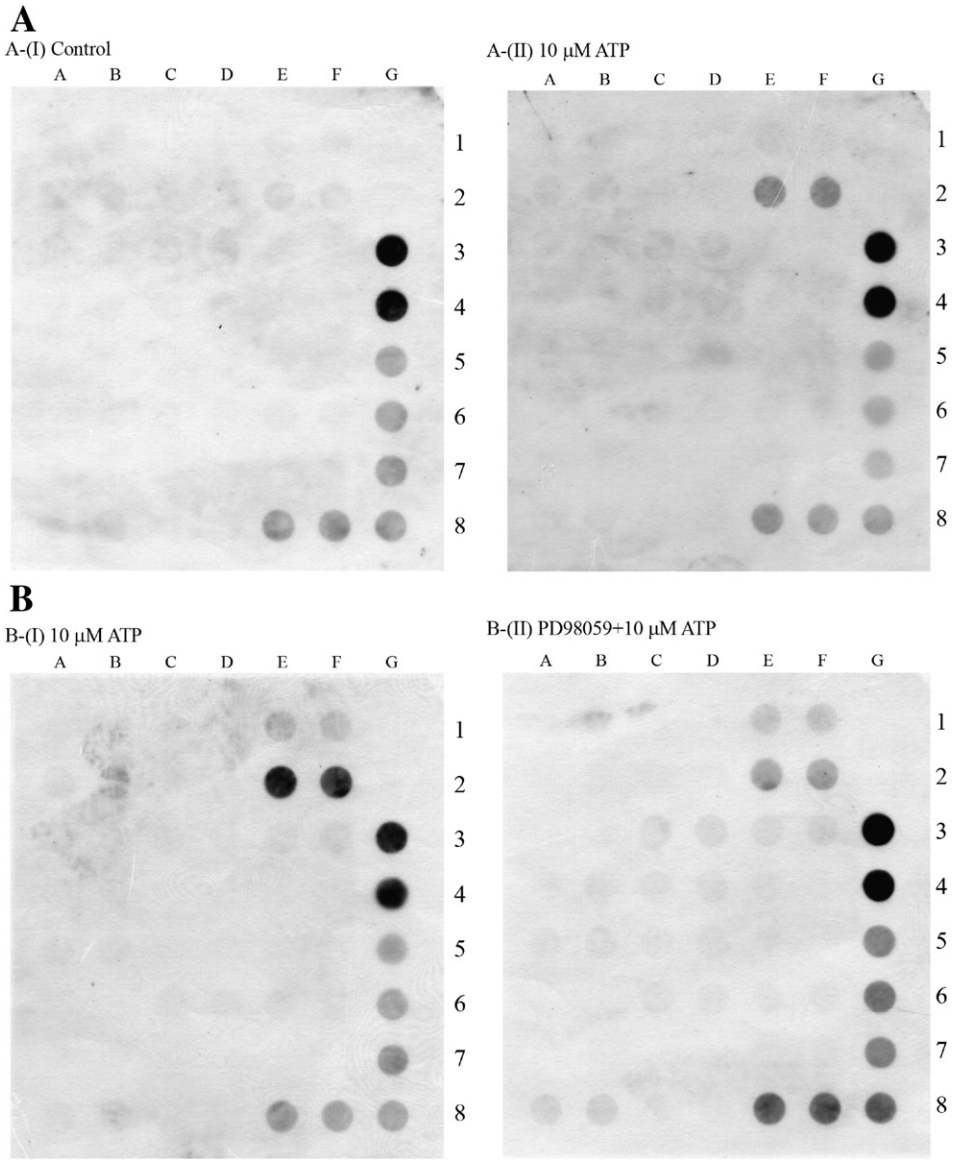

Fig. 5. (A) The effects of ATP on gene expression (2E and 2F, early growth response 1 5G, 6G, 7G, 8E, 8F, and 8G, GAPDH; 3G and 4G, β actin). (B) The effect of PD98059 on ATP-induced gene expression. (C) The time effects of ATP on gene expression. (C-I) Control group. (C-II) Human ESCs were treated with 10 μM ATP for 30 min (1e, egr-1; 7 m and 8 m, GAPDH). (C-III) Human ESCs were treated with 10 μM ATP for 24 h. The data are shown as the relative ratio to GAPDH levels. Values are presented as the mean ± SE of 3 different patients.

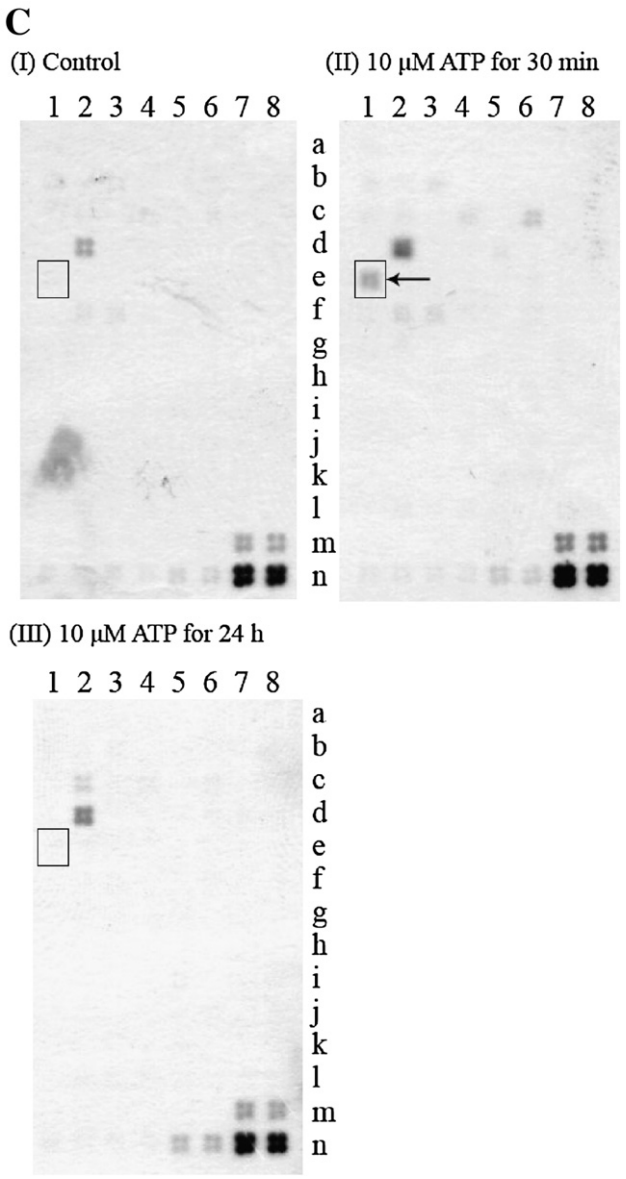

Fig. 5 (*continued*).

10 μM. Separately, a remarkable effect was seen within 5 min after 10 μM ATP treatment, and the maximal effect was noted at the 10-min time-point. In hGLCs, ATP induced nuclear translocation of phospho-ERK1/2 after treatment for 5 min [31]. In hESCs, ATP induced nuclear translocation of phospho-ERK1/2 was seen after treatment for 10 min. In terms of gene expression, ATP activates ERKs leading to the induction of egr-1 and Raf expression in hGLCs. On the other hand, exogenous ATP induced only the expression of egr-1 in hESCs. These interesting findings support the concept that ATP may exert various effects and play multi-functional roles in the human reproductive system.

In human mesenchymal stem cells, ATP modulated cell proliferation and elicited two different electrophysiological responses [32]. It was reported that extracellular ATP suppressed the growth and induced differentiation in human HL-60 leukemia cells [33]. Actually, this is the first demonstration that ATP inhibited cell viability via P2Y2 in human endometrial cells. We found no reports about the physiological relevance of P2Y2 expression in endometrial stromal cells. In the present study, we indicated that ATP inhibited cell viability through P2Y2 receptor/PLC/PKC/ERKs signaling pathway in hESCs.

5. Conclusions

To our knowledge, this is the first demonstration of the ATP-activated ERK1/2 signaling pathway, the induction of egr-1 expression and the inhibition of cell viability in human endometrial stromal cells. These results support the notion that the MAPK signaling

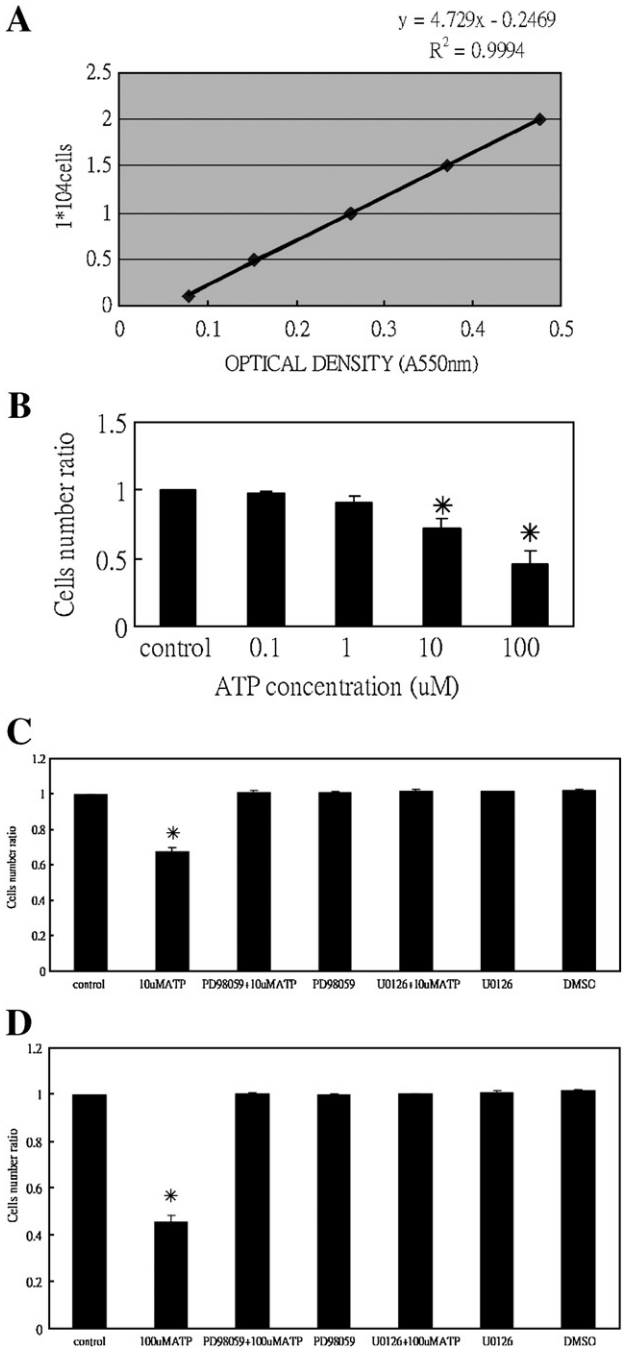

Fig. 6. The effects of ATP on cell viability. (A) The relationship between cell number and absorbance (O. D) for hESCs at a range of $(0.1–2.0)\times 10^4$ cells per well in 100 μL medium. (B) Human ESCs were treated with increasing concentrations of ATP (0, 0.1, 1, 10, 100 μM), and numbers of hESCs at 1×10^4 cells per well in 100 μL medium were measured using an MTT assay 24 h after administration, as described in Materials and methods. (C) The inhibition of 10 μM ATP on cell viability was blocked in the presence of PD98059 (50 μM) or U0126 (50 μM) in hESCs. (D) The inhibition of 100 μM ATP on cell viability was blocked in the presence of PD98059 or U0126 in hESCs. The data are shown as relative ratios to basal levels. Values are presented as the mean±SE of 3 different patients. Statistical analysis was performed by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered significant at $p<0.05$, *.

pathway plays a role in mediating ATP action in the human reproductive system.

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