Extracellular ATP activates nuclear translocation of ERK1/2 leading to the induction of matrix metalloproteinases expression in human endometrial stromal cells

Shu-Ju Chang[1], Tao-Yeuan Wang[2], Yi-Hsuan Lee[3] and **Chen-Jei Tai[4]**

[1]Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan
[2]Department of Pathology, Mackay Memorial Hospital and Mackay Medicine, Nursing and Management College, Taipei, Taiwan ROC
[3]Department of Physiology and Department of Traditional Chinese Medicine, Taipei Medical University Hospital, Taipei, Taiwan ROC
[4]Department of Obstetrics and Gynecology, Taipei Medical University, 252 Wu-Xing Street, Xin-Yi District, Taipei, 110, Taiwan ROC
(Requests for offprints should be addressed to C-J Tai; Email: chenjtai@tmu.edu.tw)

Abstract

ATP has been shown to activate the mitogen-activated protein kinase (MAPK) signaling pathway in various systems. However, little is known about the signaling events and the effects in human endometrial stromal cells (hESCs). The present study examined the effect of ATP on activating MAPKs and its subsequent events in hESCs. This study demonstrated the expression of the P_{2U}/$P2Y_2$ receptor in hESCs by reverse transcription-PCR (RT-PCR). A PCR product with a sequence identical to the reported 599 bp P_{2U}/$P2Y_2$ receptor cDNA was obtained. Western blot analysis, using a monoclonal antibody against the phosphorylated forms of ERK1/2, demonstrated that ATP activated MAPK in a dose- and time-dependent manner. Confocal microscopy showed an evident nuclear translocation of phosphorylated ERKs after 10 μM ATP treatment, but this effect was blocked by PD98059. To study the gene(s) induced by exogenous ATP, mRNA was extracted from hESCs in the presence or absence of 10 μM ATP. The gene array for 96 genes associated with members of human matrix metalloproteinases (MMPs) and adhesion molecules revealed that the expression of *MMP-2*, *-3*, *-10*, and *-24* genes was increased and the effect was attenuated by PD98059. Furthermore the effects of ATP on the expression of *MMP* genes were confirmed by semiquantitative RT-PCR. To our knowledge, this is the first demonstration of the ATP-induced nuclear translocation of phospho-ERK1/2 that mediates *MMPs* gene expression in human endometrial cells. These results support the notion that the ERK1/2 signaling pathway is involved in mediating ATP actions in the human reproductive system.

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Introduction

The female reproductive system is innervated by autonomic nerves and supplied by blood vessels (Willson *et al.* 1965, Owman *et al.* 1967). ATP is released from cells such as platelets and co-released with neurotransmitter granules from autonomic nerve endings by exocytosis (Gordon 1986). Extracellular ATP binds to a G-protein-coupled P2 purinoceptor and induces intracellular signaling transduction (Berridge 1984, Gordon 1986). Thereafter, ATP may participate in various types of physiological responses, including intracellular signaling transduction, secretion, cell proliferation, platelet aggregation, and neurotransmission (el–Moatassim *et al.* 1992, Burnstock & Knight 2004).

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 functions such as implantation, cell proliferation, and cell differentiation. Our previous data demonstrated that extracellular ATP, through a transmembrane purinergic receptor (Tai *et al.* 2000), plays a crucial role in regulating ovarian functioning by activating the intracellular signaling pathway and modulating human chorionic gonadotropin action (Tai *et al.* 2001*a*,*b*). However, the role of ATP in other human reproduction-related cells, such as human endometrial stromal cells (hESCs), remains to be determined. The present study was designed to examine the presence of the purinergic receptor and the effects of exogenous ATP on the intracellular mitogen-activated protein kinases (MAPKs) and gene expression in hESCs.

MAPKs are a group of serine–threonine kinases involved in relaying extracellular stimuli into intracellular signals (Brunet & Pouyssegur 1997). When activated, ERK1 and ERK2 (also known as $p42^{mapk}$ and $p44^{mapk}$ respectively) may be imported into the nucleus and phosphorylate a variety of substrates, which have been implicated in the control of cell

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proliferation, differentiation, and gene expression (Brunet *et al.* 1999, Kim-Kaneyama *et al.* 2000). The present study was designed to examine the action of ATP on nuclear translocation of activated ERK1/2 in hESCs.

Tissue remodeling involving endometrial extracellular matrix (ECM) turnover plays a major role in placenta invasion, regulated by the combined action of matrix metalloproteinases (MMPs) and the tissue inhibitors of MMPs (TIMPs). The MMPs are a family of extracellular endopeptidases that selectively degrade components of the ECM (Henriet *et al.* 2002). Based on substrate specificity, the MMPs are grouped into: 1) collagenases, which regulate interstitial ECM turnover by degrading interstitial collagens types I–III via a specific cleavage that denatures the helical structure of these fibrillar collagens; 2) gelatinases, which degrade basement membrane collagens IV and V as well as denatured interstitial collagens (gelatins); 3) stromelysins, which degrade such diverse ECM proteins as proteoglycans, glycoproteins, fibronectin, and laminin, and can cleave the globular domain of interstitial (type III) and basement membrane collagen types IV and V; and 4) membrane-type MMPs, which digest a number of ECM molecules. MT1-MMP has collagenolytic activity on types I–III collagens (Dong *et al.* 2002). One of the striking features of the MMPs is that many of those genes are 'inducible'. The activators include growth factors, cytokines, and chemical agents. The enhanced *MMP* gene expression may be downregulated by suppressive factors such as TIMPs (Goffin *et al.* 2003, Visse & Nagase 2003). In the present study, we examined the effect of ATP on the expression of *MMP* genes in hESCs.

Materials and Methods

Reagents and materials

ATP was obtained from Sigma Chemical Co. PD98059, a MEK inhibitor, was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from GIBCO-BRL. PD98059 was dissolved in dimethyl sulfoxide, 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. Normal donkey serum, biotin-SP-conjugated AffiniPure (goat anti-rabbit IgG (H+L) or goat anti-mouse IgG (H+L)), Cy2-conjugated streptavidin and Cy3-conjugated streptavidin were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). TOTO-3 iodide was bought from Molecular Probes, Inc. (Eugene, OR, USA). BSA and glycerol were obtained from Sigma Chemical Co. GEArray was purchased from SuperArray Bioscience Corporation (Frederick, MD, USA; www.superarray.com; GEArray Q Series Human ECM & Adhesion Molecules Gene Array). Access RT-PCR System was obtained from Promega Corporation (Cat. no. A1250; www.promega.com).

Tissue collection and hESCs cultures

Samples of proliferative phase endometrium were obtained from 30 cyclic 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 protocol reported by Shiokawa *et al.* (1996) and Gargett *et al.* (2001). Briefly, the 100 mg endometrium was minced and subjected to 0·1% collagenase (type IV, Sigma Chemical Co.) and 0·1% hyaluronidase (type I-S, Sigma Chemical Co.), 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. The cell pellet was 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.

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 (100 nM, 1 μM, 10 μM, or 100 μM) for 5 min. For time-course experiments, hESCs were treated with 10 μM ATP for 1, 5, 10, or 20 min. To study the action on the expression of genes of the MMPs induced by ATP, hESCs were treated with 10 μM ATP for 24 h, and the mRNA was extracted.

Total RNA isolation

Total RNA was isolated using an RNeasy Mini Kit (Qiagen GmbH). Briefly, cells were disrupted in a buffer containing guanidine isothiocyanate and homogenized following the manufacturer's protocol. Ethanol was then added to the lysate, creating conditions that promote the selective binding of RNA to the RNeasy silica-gel membrane. 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.

RT-PCR

One microgram of total RNA obtained from hESCs was reverse transcribed into cDNA using a First Strand cDNA Synthesis Kit (Pharmacia Biotech, Morgan, Canada). One set of oligonucleotide primers (5′- CCTGGAATGCGTCCACCA- CATAT-3′ and 5′- GACGTGGAATGGCAGGAAGCAGA -3′), based on the published human P_{2U}/$P2Y_2$ receptor sequence (Parr *et al.* 1994, Burnstock & Knight 2004) was designed for PCR to amplify the P_{2U}/$P2Y_2$ from hESCs. PCRs were performed in the presence of 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 400 μm dNTPs, 0·25 U Taq DNA polymerase, 2 μM primers, and 1 μl cDNA template per 25 μl reaction. Amplification was carried out for 33 cycles with denaturation at 94 °C for 60 s, annealing at 64 °C for 35 s and extension at 72 °C for 90 s, and a final extension at 72 °C for 15 min. Ten microlitres of the PCR products of P_{2U}/$P2Y_2$ receptor were fractionated in a 1% agarose gel stained with ethidium bromide. The expected PCR products (599 bp) were isolated from the gel, cloned using a TA cloning kit (Invitrogen), and sequenced by the dideoxy chain termination method using a T7 DNA polymerase sequencing kit (Pharmacia Biotech). The sequence of the cDNA was sent to the GenBank at the National Center for Biotechnology Information (NCBI) through the internet (www.ncbi.nlm.nih.gov), in order to compare the identity with published human P_{2U}/$P2Y_2$ receptor.

To examine the dose-effect of ATP on MMPs mRNA, hESCs were treated with increasing concentrations of ATP (0·1 μM, 10 μM, or 100 μM) for 24 h, and the mRNA was extracted. One microgram total RNA was reverse transcribed into first strain cDNA using the Access RT-PCR System according to the manufacturer's instructions (Promega). Primers for human MMP-2 (5′-AGATCTTCTTCTTCA- AGGACCGGTT-3′ and 5′-GGCTGGTCAGTGGCTTGGGGTA-3′) were designed based on published sequences (Goffin *et al.* 2003). Primers for human MMP-3 (5′-GAAATTCCA- TGGAGC-CAGGG-3′ and 5′-AGTGTGACTCGAGTCACAGC- 3′) and primers for human MMP-10 (5′-GCAGCGGACAAA-TACTGGAGA-3′ and 5′-TATGTGTGT- CACCAT-CCTGGC-3′) were designed based on published sequences (Kim *et al.* 2001). Primers for human MMP-24 (5′-CACAAGGCCACT- CCCTACAC-3′ and 5′-TAGGTCT TG- CCCACAGGTTC-3′) were designed based on published sequences (Jung *et al.* 2003). The one-step RT-PCR conditions were 45 °C for 45 min to synthesize the first strand of cDNA, 94 °C for 2 min to denature the template and to synthesize the second strand and DNA amplification. Amplification of MMP-2 was carried out for 27 cycles with denaturation at 94 °C for 15 s, annealing at 68·5 °C for 20 s, and extension at 72 °C for 10 s, and a final extension at 72 °C for 2 min. Amplification of MMP-3 was carried out for 35 cycles with denaturation at 94 °C for 40 s, annealing at 56·1 °C for 90 s, and extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. Amplification of MMP-10 was carried out for 30 cycles with denaturation at 94 °C for 40 s, annealing at 57·7 °C for 90 s, and extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. Amplification of MMP-24 was carried out for 45 cycles with denaturation at 95 °C for 1 s, annealing at 66·4 °C for 5 s, and extension at 72 °C for 15 s, and a final extension at 72 °C for 7 min. The PCR annealing temperature was selected by the Mastercycler gradient 5331 machine (Eppendorf AG, Hamburg, Germany). In addition, GAPDH of the individual sample was used as the internal control. Primers for GAPDH (5′-ATGTTCGTCATGGGT- GT-GAACCA-3′ and 5′-TGGCAGGTT- TTTCTAGACGG-CAG-3′) were designed based on published sequences (Tokunaga *et al.* 1987). Amplification was carried out for 18 cycles with denaturation at 94 °C for 60 s, annealing at 57·1 °C for 35 s, and extension at 72 °C for 90 s, and a final extension at 72 °C for 15 min. Eight microliters of PCR products of MMP-2, -3, -10, and -24 were fractionated in a 3% agarose gel and stained with ethidium bromide for 30 min and visualized under a u.v. transilluminator (E-BOX 1000, Vilber Lourmat, Marne-la-Vallee, France). A 100 bp DNA ladder (Promega) was used as a molecular weight marker. The autographs were quantified with E-CAPT software (Vilber Lourmat).

Western blot analysis

The hESCs were washed with ice-cold PBS and lysed with 100 μl cell lysis buffer (RIPA, 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), following the manufacturer's protocol. Aliquots (30 μg) were subjected to 10% SDS–PAGE under reducing conditions as previously described (Laemmli 1970). The proteins were electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Pharmacia Biotech), following the procedures of Towbin *et al.* (1979). These nitrocellulose 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 (Cell Signaling Technology Inc.). After washing, the membranes were incubated with HRP-conjugated goat anti-mouse secondary antibody, and the signal was visualized using an ECL system (Amersham Pharmacia Biotech) 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).

MAP kinase assay

To measure MAP kinase activity, a nonradioactive method was used (p44/42 MAP Kinase Assay Kit, Cell Signaling Technology Inc). Briefly, active MAP kinase of cell lysate (200 μg) from hESCs treated with 10 μM ATP for 10 min was selectively immunoprecipitated with an immobilized monoclonal

antibody to phospho-p44/42 MAP kinase. For the positive control, active MAP kinase (provided by the manufacturer) was added to the control cell extract. The resulting precipitate was incubated with an Elk-1 fusion protein in the presence of ATP, which allowed immunoprecipitated active MAPK to phosphorylate Elk-1. Phosphorylated Elk-1 was detected by western blot using a phospho-Elk-1 antibody.

Confocal fluorescence microscopy

Human ESCs were plated onto glass coverslips and grown to 40% confluence at 37 °C in humidified air with 5% CO_2. Cells were treated with 10 μM ATP for 10 min in the absence or presence of PD98059 (pretreated for 30 min before ATP exposure), fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 1% Nonidet P-40 in PBS for 20 min. Nonspecific staining was blocked with 10% normal donkey serum. The cells were incubated with the primary antibody, phosphorylated ERK1/2 or total ERK1/2, diluted 1: 400 with 2% BSA in PBS overnight at 4 °C. After three washes in PBS containing 0·1 Tween-20, the cells were incubated with goat anti-mouse IgG (H+L) or goat anti-rabbit IgG (H+L) for 60 min at room temperature, and washed as above. The cells were then incubated with Cy2-conjugated streptavidin or Cy3-conjugated streptavidin, diluted in the ratio of 1: 3000 in PBS, for 30 min at room temperature. After the antibody incubations, the coverslips were washed in PBS, and nuclei were stained with TOTO-3 iodide, diluted in PBS, for 20 min at room temperature. Coverslips were mounted onto slides with glycerol and viewed on a confocal laser scanning microscopy (Model FV500, Olympus, Tokyo, Japan) using the 40× objective. Images were acquired and quantified using FLUOVIEW software (version 4.0, Olympus).

Gene array analysis

Human ESCs were treated with 10 μM ATP for 24 h in the absence or presence of PD98059 (pretreated for 30 min before ATP exposure) prior to RNA extraction. Biotinylated cDNA probes were synthesized from 5 μg total RNA of ATP-treated or control samples using SuperArray's proprietary GEAprimer mix as reverse transcriptase primers and hybridized to the GEArray Q Series membrane spotted with 96 gene-specific cDNA fragments, following the manufacturer's instructions (SuperArray Bioscience). Briefly, 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, Bioscience). Annealing of RNA with primers was performed in a preheated heat block 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. For cDNA probe synthesis, each RNA sample (5 μg) was combined with a primer mix and with reverse transcriptase, RNase inhibitor, and a dNTP mix with biotin-16-dUTP and incubated at 42 °C for 90 min. The GEArray membrane spotted with 96 gene-specific cDNA fragments was moistened with deionized H_2O, 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 2× SSC containing 1% SDS for 20 min at 60 °C, and twice with pre-warmed 0·1× SSC containing 0·5% SDS for 20 min at 60 °C. After blocking with GEAblocking solution, the membrane was 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 of 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 GAPDH. Images of the membranes were recorded on X-ray film and the intensity of the array of

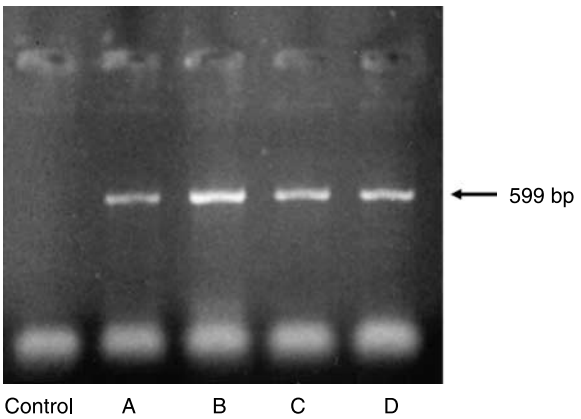
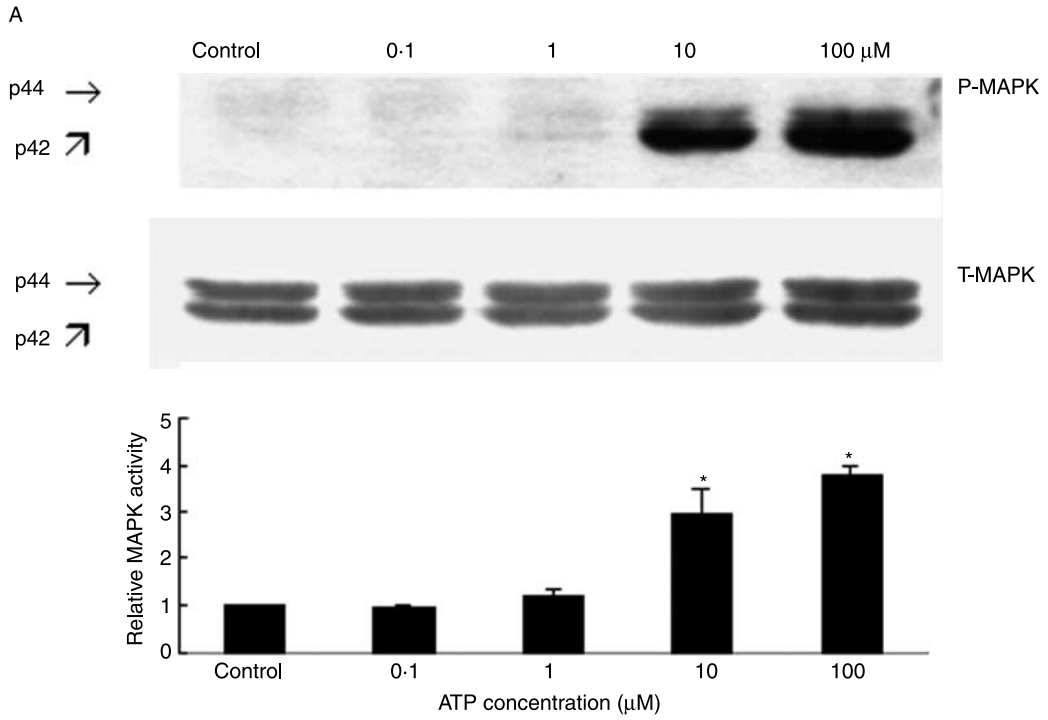


Figure 1 Ethidium bromide-stained DNA gels showing the PCR products from three patients. Columns A–C are PCR products of endometrial stromal cells. Column D represents as positive control obtained from human granulosa luteal cells. One microgram total mRNA of hESCs from each patient was reverse transcribed into cDNA, and aliquots were amplified using PCR. A 599 bp product was obtained. The control did not have first strain cDNA in the PCR.

Figure 2. (A) The dose-response of ATP on MAPK activation in hESCs. Human ESCs were treated with increasing concentrations of ATP (0, 0·1 μM, 1 μM, 10 μM or 100 μM) for 5 min as described in Materials and Methods. (B). The time course of ATP on MAPK activation in hESCs. Human ESCs were treated with 10 μM ATP for 0, 1, 5, 10 or 20 min, as described in Materials and Methods. The MAPKs were detected by western blot analysis. The data are shown as the relative ratio to basal levels. Values are presented as the mean±S.E.M. of three different patients. Differences were considered significant at $^*P<0·05$.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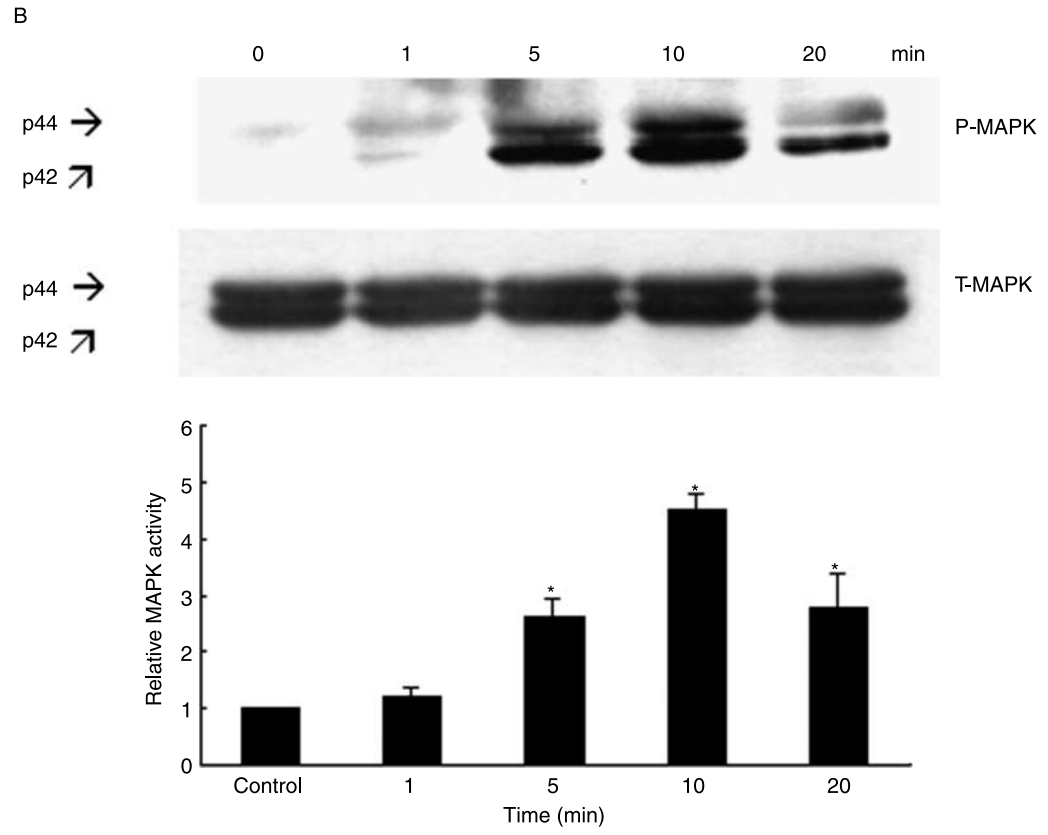
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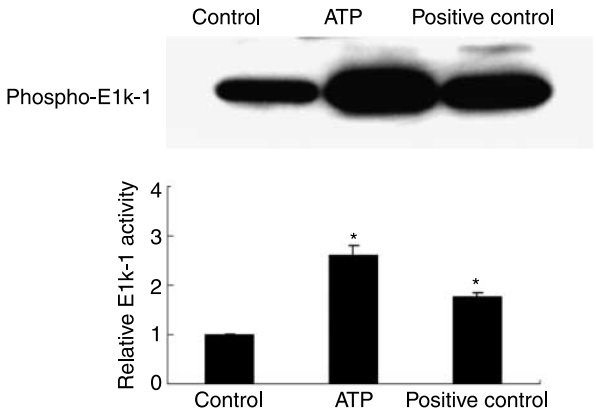


Figure 3 MAP kinase activity in hESCs measured using a MAP kinase assay kit. Human ESCs were treated with 10 μM ATP for 10 min, as described in Materials and Methods. Active p42 MAPK was included as a positive control. Values are presented as the mean±S.E.M. of three different patients. Differences were considered significant at $*P<0{\cdot}05$.

spots was converted into Adobe Photoshop as a TIFF file. The signal intensity of the spots was compared using the GEArray Expression Analysis Suite (SuperArray Corp., http://www.superarray.com).

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 three different patients. Data were represented as means±S.E.M. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at $P<0{\cdot}05$.

Results

Expression of P_{2U}/P2Y$_2$ receptor mRNA in hESCs

The expression of the P_{2U}/P2Y$_2$ receptor mRNA in hESCs was examined by RT-PCR using one set of primers designed on the basis of the published human P_{2U}/P2Y$_2$ receptor expressed in airway epithelium. An expected 599-bp DNA fragment was observed in ethidium bromide-stained gel from hESCs isolated from three different patients (Fig. 1). No product was obtained from the negative control (without a first strain cDNA template in the PCR). The PCR products from hESCs were subcloned and sequenced. Sequence analysis revealed that the cloned cDNA was identical to nucleotide position 436–1034 of the published human P_{2U}/P2Y$_2$ receptor (Parr *et al.* 1994, Burnstock & Knight 2004).

Effect of ATP on MAPK activation

To demonstrate the ability of ATP to activate MAPK, hESCs were treated with increasing concentrations (100 nM–100 μM) of ATP for 5 min. For time-course analysis, the cells were treated with 10 μM ATP for varying time intervals (1–20 min). As shown in Fig. 2A, 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. ATP was capable of rapidly inducing MAPK activity. 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 its maximum at 10 min (Fig. 2B).

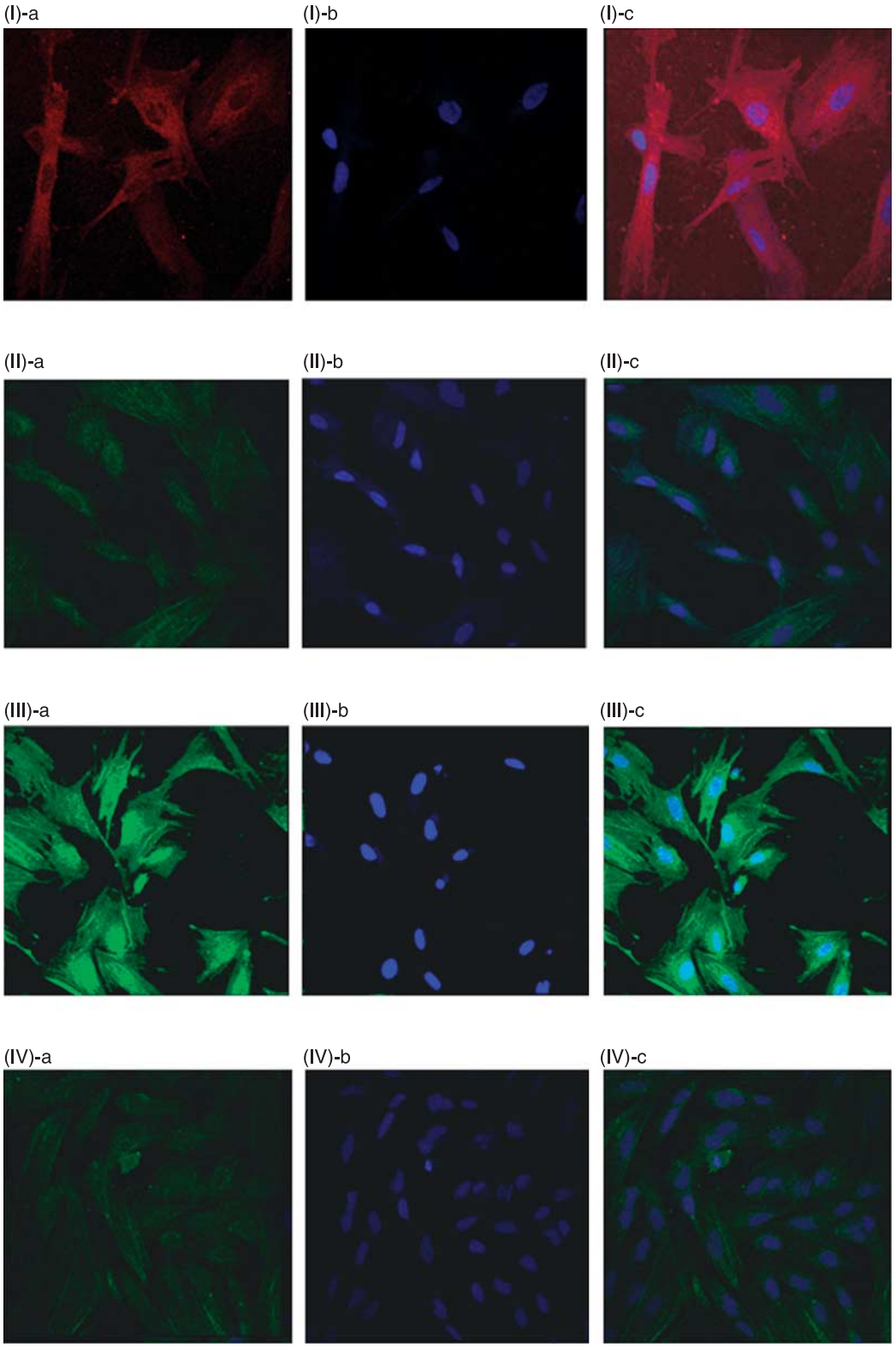
MAP kinase activity

In vitro MAP kinase activity was detected using a p44/42 MAP kinase assay kit. As shown in Fig. 3, ATP significantly increased MAPK activity to 250% of the control level.

Subcellular ERK1/2 localization

As shown in Fig. 4(I)-a, the use of a polyclonal antibody against total ERKs demonstrated that ERK1/2 (nonphosphorylated and phosphorylated) were distributed in the cytoplasm. To observe the baseline level of phosphorylated ERK1/2, cells were fixed in the absence of ATP, and the faint intracellular fluorescence revealed that small amounts of phosphorylated ERK1/2 were located in the cytoplasm (Fig. 4II-a). To examine the distribution of ATP-activated

Figure 4. The distribution of p42/p44 and the effect of ATP on phospho-p42/p44 translocation in hESCs. (I)-a, the distribution of p42/p44 in hESCs. Formaldehyde-fixed cells were incubated with polyclonal anti-p42/p44 MAPK antibody followed by goat anti-rabbit IgG (H+L) and Cy3-conjugated streptavidin. (I)-b, nuclei of cells in (I)-a were stained with TOTO-3 iodide. (I)-c, the final merged image of (I)-a and (I)-b. (II)-a, the localization of the phospho-p42/p44 in hESCs prior to ATP treatment. Formaldehyde-fixed hESCs, in the absence of ATP, were incubated with monoclonal anti-phospho-p42/p44 antibody followed by goat anti-mouse IgG (H+L) and Cy2-conjugated streptavidin. (II)-b, nuclei of cells in (II)-a were stained with TOTO-3 iodide. (II)-c, the final merged image of (II)-a and (II)-b. (III)-a, the localization of the activated p42/p44 (phospho-p42/p44) in hESCs. Human ESCs were treated with 10 μM ATP for 10 min. Formaldehyde-fixed cells were incubated with monoclonal anti-phospho-p42/p44 antibody followed by goat anti-mouse IgG (H+L) and Cy2-conjugated streptavidin. (III)-b, nuclei of cells in (III)-a were stained with TOTO-3 iodide. (III)-c, the final merged image of (III)-a and (III)-b. (IV)-a, the effect of PD98059 on ATP-induced translocation of the activated p42/p44 in hESCs. Human ESCs were treated with 10 μM ATP in the presence of PD98059 for 10 min. Formaldehyde-fixed cells were incubated with monoclonal anti-phospho-p42/p44 antibody followed by goat anti-mouse IgG (H+L) and Cy2-conjugated streptavidin. (IV)-b, nuclei of cells in (IV)-a were stained with TOTO-3 iodide. (IV)-c, the final merged image of (IV)-a and (IV)-b.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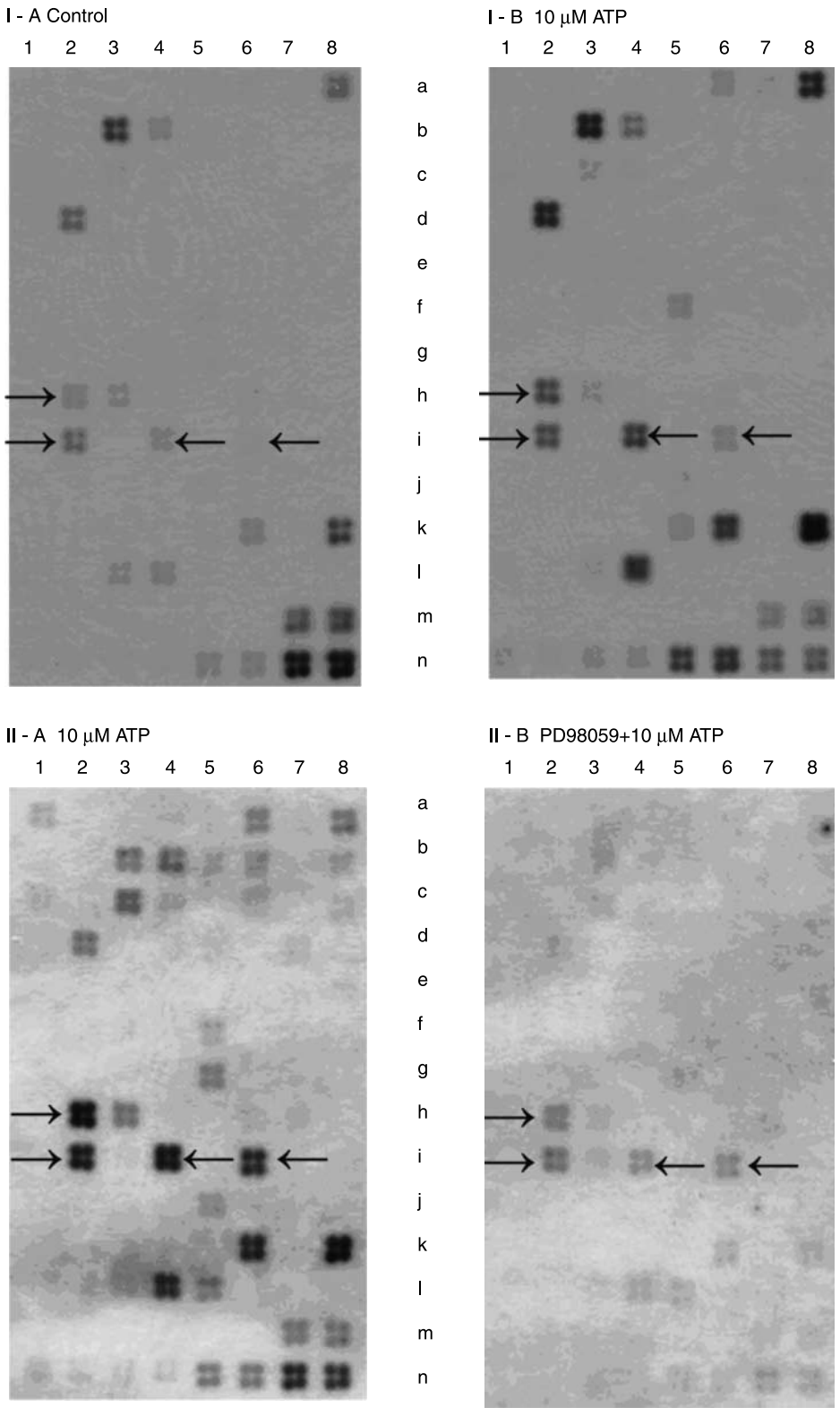
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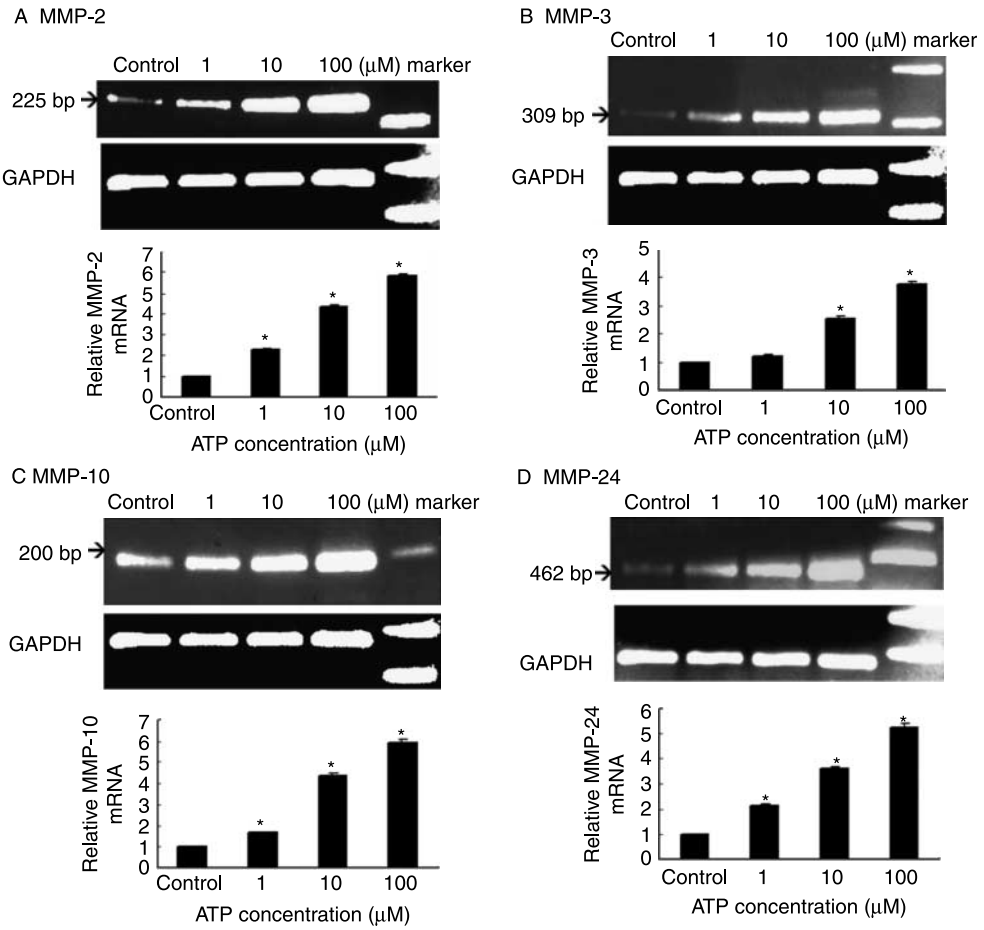

Figure 6 The effect of ATP on expression of MMP-2, -3, -10, and -24 in hESCs. Human ESCs were treated with increasing concentrations of ATP (0, 1 μM, 10 μM or 100 μM) for 24 h as described in Materials and Methods. (A) MMP-2, 225 bp, (B) MMP-3, 309 bp, (C) MMP-10, 200 bp, and (D) MMP-24, 462 bp, were separated in 3% agarose gels, independently. The data are shown as the relative ratio to basal levels. Values are presented as the mean ± S.E.M. of three different patients. Differences were considered significant at $^*P<0{\cdot}05$.

ERK1/2, hESCs were treated with 10 μM ATP for 10 min. Once activated, phosphorylated ERK1/2 were translocated into the nuclei, and were detected by a monoclonal antibody against phosphorylated ERK1/2 (Fig. 4III-a). Figure 4IV-a demonstrates that, in the presence of PD98059, the effect of ATP on ERK1/2 translocation was completely blocked. A faint fluorescent staining revealed the distribution of phosphorylated ERK1/2 in the cytoplasm. The nuclear translocation of phosphorylated ERK1/2 was not significant when compared with the cells in Fig. 4II. The nuclei of hESCs in the present study were stained with TOTO-3 iodide and emitted blue fluorescence (Fig. 4I-b–IV-b).

Gene array analysis

Total RNA extracted from hESCs incubated in the absence or presence of 10 μM ATP for 24 h, was converted to cDNA. Superarray analysis for 96 genes related to human ECM and adhesion molecules revealed that the expression of MMP-2 (spots 2*i*), MMP-3 (spots 6*i*), MMP-10 (spots 2*h*), and MMP-24 (spots 4*i*) was increased (Fig 5-I-A and I-B). The relative abundance of MMP-2 in the ATP-treated group was 3·5-fold greater than the control group when comparing their signal intensities to the signals derived from GAPDH from three different patients. In the presence of ATP, the expression of MMP-3 was increased by 3·6-fold,

Figure 5 Gene array of human extracellular matrix and adhesion molecules described in Materials and Methods. I-(A) Control group. I-(B) Human ESCs were treated with 10 μM ATP for 24 h. After hybridization, the membrane was incubated with alkaline phosphatase-conjugated streptavidin, and the signal was visualized with CDP*Star*, a chemiluminescent substrate, and exposed to X-ray film (2*h*, MMP-10; 2*i*, MMP-2; 4*i*, MMP-24; 6*i*, MMP-3; 7*m* and 8*m*, GAPDH). As shown in II-(A) Human ESCs were treated with 10 μM ATP for 24 h. II-(B) Human ESCs were pretreated with PD98059 for 30 min prior to 10 μM ATP exposure. The data are shown as the relative ratio to GAPDH levels. Values are presented as the mean ± S.E.M. of three different patients.

MMP-10 by 4·2-fold, and MMP-24 by 3·6-fold. To examine the direct effect of MMPs in gene expression, hESCs were pretreated with PD98059 for 30 min prior to 10 μM ATP exposure, and the mRNA was extracted. As shown in Fig. 5-II-A and II-B, the effects of ATP on the expression of MMP-2, -3, -10, and -24 was significantly downregulated in the presence of PD98059.

Effect of ATP on MMP-2, -3, -10, and -24 expressions

To demonstrate the ability of ATP in regulating *MMP*-2, -3, -10, and -24 genes, hESCs were treated with increasing concentrations (0, 1 μM, 10 μM, or 100 μM) of ATP for 24 h. As shown in Fig. 6A, ATP upregulated the level of expression of MMP-2 mRNA in a dose-dependent manner. A significant effect was observed at 1 μM ATP, with a maximum effect noted at 100 μM. The relative abundance of MMP-2 in the ATP-treated group was 2·2-fold, 4·4-fold, and 5·8-fold greater than the control level. As shown in Fig. 6B, ATP upregulated the level of expression of MMP-3 mRNA in a dose-dependent manner. A significant effect was observed at 10 μM ATP, with a maximum effect noted at 100 μM. The relative abundance of MMP-3 in the ATP-treated group was 1·2-fold, 2·6-fold, and 3·7-fold greater than in the control group. As shown in Fig. 6C, ATP upregulated the level of expression of MMP-10 mRNA in a dose-dependent manner. A significant effect was observed at 1 μM ATP, with a maximum effect noted at 100 μM. The relative abundance of MMP-10 in the ATP-treated group was 1·7-fold, 4·3-fold, and 5·9-fold greater than the control level. As shown in Fig. 6D, ATP upregulated the level of expression of MMP-24 mRNA in a dose-dependent manner. A significant effect was observed at 1 μM ATP, with a maximum effect noted at 100 μM. The relative abundance of MMP-24 in the ATP-treated group was 2·1-fold, 3·6-fold, and 5·2-fold greater than in the control group.

Discussion

These results demonstrate for the first time that P_{2U}/$P2Y_2$ receptor mRNA is expressed in hESCs, and that ATP is able to activate ERK1/2, induce the nuclear translocation of phosphorylated ERKs, and increase the expression of MMP-2, -3, -10, and -24 in hESCs.

Purinergic receptors have been classified as P1 and P2 receptors. Pharmacological study demonstrated that P1 receptors have a high affinity for extracellular adenosine (Adenosine > AMP > ADP > ATP), whereas P2 receptors have a high affinity for ATP (ATP > ADP > AMP > adenosine). P2 purinergic receptors, $P2X_{1\text{-}7}$, $P2Y_{1,2,4,6,11,12,13,14}$ have been identified in molecular cloning studies (Gordon 1986, Burnstock & Knight 2004, Burnstock 2006), and the P_{2U}/$P2Y_2$ purinergic receptor has an equal or higher response to UTP than to ATP. The demonstration of the P_{2U}/$P2Y_2$ purinergic receptor in hESCs implies that ATP may play a role in coordinating reproductive functions, such as the menstrual cycle and embryo implantation.

MAP kinases have been identified and they play important roles in the reproductive system (Zhang *et al.* 2002). The MAP kinases have been implicated in the regulation of cell growth and differentiation (Fanger 1999). MAP kinases are classified into three subfamilies: a) ERKs (extracellular signal-regulated kinases), including ERK1 and ERK2, b) SAPKs (stress-activated protein kinases), also called c-jun N-terminus kinases (JNKs), and c) p38 kinase (Tai *et al.* 2001*a*). The first MAPKs to be cloned were MAPK/ERK 1 and 2. They are phosphorylated and activated by MEKs (Boulton *et al.* 1991). Our results demonstrated that ATP is able to activate ERK1/2 in a dose- and time-dependent manner. Additionally, we demonstrated that ATP is capable of inducing the translocation of phosphorylated ERK1/2 from the cytoplasm to the nucleus in hESCs. The current study further examined the intracellular functioning of activated ERK1/2 in hESCs.

Extracellular ATP binds to a purinergic receptor, which belongs to one of the G-protein-coupled receptors (GPCRs). The GPCRs are used to control the activity of MAP kinases. ERK activation occurring via the GPCR/PKC pathway and EGF receptor transactivation lead to the nuclear translocation of the kinases and stimulate cell proliferation (Brunet *et al.* 1999, Luttrell 2002). When activated, ERKs phosphorylate a variety of substrates in the nucleus, including transcription factors and gene transcription, which have been implicated in the control of cell proliferation, differentiation and gene expression (Brunet *et al.* 1999). It is believed that the nucleus is also a significant site for mitogenic signal termination by the nuclear sequestration of p42/p44 MAPKs away from their cytoplasmic activator, MEK, and dephosphorylation by certain nuclear phosphatases (Volmat *et al.* 2001). In the present study, the MAP kinase assay demonstrated that ERK1/2 activated by extracellular ATP are functional by phosphorylating the potential intranuclear substrate Elk-1. Ten micromolar ATP-induced nuclear translocation of activated ERK1/2. Phosphorylated ERK1/2 play a major role in converting mitogenic stimuli into nuclear responses. We hereby showed that the effect of ATP on ERK1/2 translocation was blocked in the presence of PD98059. This suggests that the MEK activity is essential to maintain phospho-ERK1/2 accumulated in the nucleus. Previously, we demonstrated that ATP was able to activate nuclear translocation of ERK1/2 and the induction of egr-1 and c-raf-1 expression in human granulosa-luteal cells (hGLCs; Tai *et al.* 2004).

MMPs are synthesized by connective tissue cells residing in the stroma of reproductive organ. Stroma can promote epithelial development or differentiation in the female reproductive tract (Hulboy *et al.* 1997). It has been reported that the expression of MMP-2, MMP-3, and MMP-10 has been identified in hESCs during the menstrual cycle (Henriet *et al.* 2002). In the present study, we examined the effect of ATP on 96 genes associated with members of human ECM and adhesion molecules. These functional gene groupings

include cell adhesion molecules, ECM proteins, proteases, and protease inhibitors. *MMP* genes include MMP-1 (collagenase-1), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), MMP-10 (stromelysin-2), MMP-11 (stromelysin-3), MMP-12 (macrophage elastase), MMP-13 (collagenase-3), MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MMP-17 (MT4-MMP), MMP-20 (enamelysin), MMP-24 (MT5-MMP), and MMP-26. Among these genes, the expression of *MMP-2*, *MMP-3*, *MMP-10*, and *MMP-24* genes were elevated significantly (Fig. 5-I, -II). In addition, the effects of ATP on the expression of *MMP-2*, *MMP-3*, *MMP-10*, and *MMP-24* genes were confirmed in a dose-dependent study (Fig. 6 A–D) using semiquantitative RT-PCR.

MMP-2 (gelatinase A) forms the group of gelatinases that bind to and further degrade collagen denatured after initial cleavage by collagenase. In human decidual stromal cells, GnRH was capable of increasing MMP-2 and MMP-9 mRNA levels in a dose-dependent manner (Chou *et al.* 2003). It has been reported that MMP-2 may be involved in the regression of the right Müllerian duct in female chicken embryos (Ha *et al.* 2004).

MMP-3 (stromelysin 1) belongs to the group of stromelysins that degrade many components of the basement membranes and the protein core of proteoglycans. In general, MMP-3 has a proteolytic efficiency higher than that of MMP-10. In baboon endometrium, expression of stromal MMP-3 and epithelial MMP-7 increased during early menses showing that differential regulation of MMP-3 and MMP-7 is specific to cell type and stage of the menstrual cycle (Cox *et al.* 2000). It has been reported that early embryo-endometrial signaling modulates the regulation of MMP-3 in human stromal cell culture after progesterone stimulation (Lahav-Baratz *et al.* 2004).

MMP-10 (stromelysin 2) belongs to the group of stromelysins that degrade many components of the basement membranes and the protein core of proteoglycans. It has been reported that MMP-10 was found overexpressed in invasive human cervical cancer biopsies (Vazquez-Ortiz *et al.* 2005).

MMP-24 (MT5-MMP) is a member of the membrane-associated MMPs that are capable of activating proMMP-2. It has been reported that all six MT-MMPs were expressed in endometrium cells in a cycle-dependent pattern and the vascular expression of MT2-, MT3-, and MT4-MMP correlated with angiogenic events in the cycle (Plaisier *et al.* 2006). In this study, we demonstrate that ATP induced P_{2U}/$P2Y_2$ receptor leading to the expression of MMP-2, MMP-3, MMP-10, and MMP-24 in hESCs.

We reported previously the effects of extracellular ATP on *MAPKs* and gene expression in hGLCs (Tai *et al.* 2001*b*, 2004). When compared with the present 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 noted at 10 μM, and there was no statistical difference between cells treated with 10 and 100 μM ATP. In a time-dependent study, ATP was capable of rapidly inducing ERK1/2 activity. A significant effect was seen within 5 min after treatment, and the maximal effect was noted at the 5 min time-point. In contrast, no effect of ATP on ERK1/2 activation was observed at 1 μM in the hESCs. A significant effect of ATP on ERK1/2 activation was observed at 10 μM. Additionally, a significant effect was seen within 5 min after treatment with 10 μM ATP, and the maximal effect was noted at the 10 min time-point. ATP induced nuclear translocation of phospho-ERK1/2 after treatment for 5 min in hGLCs (Tai *et al.* 2004). On the other hand, ATP induced nuclear translocation of phospho-ERK1/2 was seen after treatment for 10 min in hESCs.

To our knowledge, this is the first demonstration of ATP-induced nuclear translocation of phospho-ERK1/2 and upregulation of the expression of MMP-2, -3, -10, and -24 genes in hESCs. These results support the notion that the ERK1/2 signaling pathway and MMP family play important roles in mediating ATP action in the human reproductive system.

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