Abnormal activation of Ras/Raf/MAPK and RhoA/ROCKII signalling pathways in eutopic endometrial stromal cells of patients with endometriosis

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BACKGROUND: Enhanced proliferation and survival of eutopic endometrial cells from patients with endometriosis compared with healthy women is associated with abnormal activation of extra-cellular signal-regulated kinases 1 and 2 (ERK1/2). Given the role of Ras/Raf/mitogen-activated protein kinase (MAPK) and RhoA/ROCKII signalling pathways in the regulation of cell proliferation and migration, we analysed their possible roles in endometriosis.

METHODS: Primary eutopic endometrial stromal cells of patients with endometriosis (Eu-hESC, n = 16) and endometriosis-free controls (Co-hESC, n = 14) were harvested and subjected to proliferation and migration assays as well as kinase activity assays and immunoblot analysis of proteins from the Ras/Raf/MAPK and RhoA/ROCKII signalling pathways. Effects of ROCKII (Y-27632) and MAPK (U0126) inhibitors or siRNA knockdown of ROCKII, Raf-1 and B-Raf were analysed.

RESULTS: The proliferation rate of Eu-hESC was 54% higher than Co-hESC. Eu-hESC also displayed a 75% higher migration rate than Co-hESC. Eu-hESC displayed higher levels of ERK phosphorylation (83%) and p27 expression (61%) and lower levels of Raf-1 protein (47%) compared with controls. In addition to an inhibitory effect on cell proliferation, ROCKII knockdown led to significant down-regulation of cyclinD1 and p27 but did not affect ERK phosphorylation. Down-regulation of Raf-1 by siRNA was dispensable for cell proliferation control but led to an increase in ROCKII activity and a decrease in cell migration. B-Raf was shown to act as a regulator of hESC proliferation by modulating cellular ERK1/2 activity and cyclinD1 levels. Eu-hESC displayed 2.4-fold higher B-Raf activity compared with Co-hESC and therefore exhibit abnormally activated Ras/Raf/MAPK signalling.

CONCLUSIONS: We show that the same molecular mechanisms operate in Co- and Eu-hESC. The differences in cell proliferation and migration between both cell types are likely due to increased activation of Ras/Raf/MAPK and RhoA/ROCKII signalling pathways in cells from endometriosis patients.

Key words: cell proliferation / cell migration / Ras/Raf/MAPK signalling / RhoA/ROCKII signalling / endometriosis

Introduction

Endometriosis is an oestrogen-dependent disease affecting 6–10% of women in reproductive age. It is characterized by the presence of uterine endometrial tissue outside the uterine cavity (Bulun, 2009). Susceptibility to endometriosis is thought to depend on the complex interaction of genetic, immunologic, hormonal and environmental factors. Therefore, changes in normal cellular functions such as cell proliferation, cell migration, invasiveness and cell survival could be involved in establishment of the disease.

Cell proliferation is a tightly controlled process in non-transformed cells consisting of complex levels of regulation that ensure the progression through the cell cycle. As a result, cell proliferation is normally limited to specific conditions that include stimulation by growth factors, hormones, adhesion to the extra-cellular matrix and extra-cellular matrix-specific signals. These signals need to be integrated by diverse signalling pathways to trigger an appropriate biological cellular response.

The Ras/Raf/mitogen-activated protein kinase (MAPK) signalling cascade is one of the key signalling cascades that integrates...
extra-cellular signals. The extra-cellular-signal-regulated kinases (ERK) 1 and 2 are a MAPK subfamily, and are activated by phosphorylation in response to different stimuli (Johnson and Lapadat, 2002; Roux and Blenis, 2004). ERK localization is predominantly cytoplasmic but undergoes nuclear translocation upon stimulation. However, in the presence of growth factors, translocation of activated ERK to the nucleus requires adhesion and an intact cytoskeleton to be able to induce cyclinD1 expression, critical for early checkpoint regulation at the G1 phase of the cell cycle (Aplin et al., 2001; Raman et al., 2007). The key regulators of cytoskeleton dynamics and, therefore, regulators of cell proliferation are small Rho-GTPase family proteins RhoA, Rac and Cdc42. Initial indications that Rho-GTPases contribute to cell-cycle regulation were the observations that Rho-GTPases inactivation with Clostridium botulinum C3 exoenzyme blocked serum-stimulated DNA synthesis. Moreover, microinjection of active RhoA was sufficient to induce G1/S-phase progression in Swiss 3T3 fibroblasts (Yamamoto et al., 1993; Olson et al., 1995). RhoA activates several effector proteins, with serine/threonine kinases ROCKI and ROCKII (Riento and Ridley, 2003) being the most extensively characterized. ROCKII regulates cell body contraction during migration by acting on actomyosin contractility (Fukata et al., 1999; Sumi et al., 2001). The function of ROCKII in the control of cell proliferation was shown in smooth muscle cells (Sawada et al., 2000) and hepatocytes (Iwamoto et al., 2000), where the inhibition of ROCKs was reported to up-regulate the levels of cyclin-dependent kinase inhibitor p27 and to suppress G1 to S-phase progression.

Incomplete transitioning of endometrium from proliferative (oestrogen-dependent) to secretory (progesterone-dominated) phase is a feature of eutopic endometrium of patients with endometriosis. This correlates with a phenotype of enhanced cellular survival, enhanced proliferation and reduced differentiation (decidualization) (Kao et al., 2003; Burney et al., 2007; Velarde et al., 2009). Association of MAPK activity with the pathogenesis of endometriosis was first described in ectopic lesions (Yoshino et al., 2004). Up-regulation of MAPK subfamilies are thought to play important roles in regulating growth and maintenance of eutopic endometrial tissue by influencing expression and function of various cytokines (Yamauchi et al., 2004; Wu et al., 2005). Higher cell proliferation in eutopic endometrial stromal cells from women with endometriosis (Eu-hESC) than in eutopic endometrial stromal cells from women without this disease (Co-hESC) was also proposed to correlate with up-regulation of ERK phosphorylation (Murk et al., 2008; Velarde et al., 2009). These findings emphasized the role of the Ras/Raf/MAPK signalling cascade as a regulator of cell proliferation in endometrial cells. However, it is still not known which of the Raf serine/threonine kinase isoforms is the downstream effector of Ras in endometrium. In addition, it is still poorly understood how the persistent activation of ERK is achieved in Eu-hESC and whether the RhoA/ROCKII signalling pathway is involved in this process. During the last decade, the potential role of the RhoA/ROCKII signalling pathway in the regulation of cell proliferation in hESC and its role in the development of endometriosis have not been elucidated yet.

Given the role of Ras/Raf/MAPK and RhoA/ROCKII in the regulation of cell proliferation, we hypothesize that the basal cell proliferation in Eu-hESC and Co-hESC is under the control of both signalling pathways. A recent report demonstrated a higher migration rate of Eu-hESC compared with Co-hESC both at basal conditions as well as after oestrogen stimulation (Gentilini et al., 2010). Therefore, in order to ensure high migration capacity of Eu-hESC, we further hypothesize that RhoA/ROCKII activity is abnormally regulated in Eu-hESC.

### Materials and Methods

#### Patients and tissue collection

Endometrial samples (n = 30) were obtained from patients (age 18–42) who underwent laparoscopy and additional curettage for diagnosis and/or treatment of endometriosis, chronic pelvic pain, ovarian cysts, dysmenorrhea and/or infertility (Table I). The presence or absence of endometriosis was confirmed visually by laparoscopy and additional histological analysis. In particular, eutopic endometrium samples of patients with endometriosis (n = 16) and eutopic endometrium samples of age-matched endometriosis-free controls (n = 14) were used for primary cell culture preparation. All donors were not taking medications and had not received hormonal therapy for at least 6 months prior to surgery. All samples were obtained following institutional review board and ethical committee approval (ethical committee number 075/2005 General Hospital of Vienna, Vienna, Austria) as well as written informed consent permission from every participating woman. Immediately after surgery, the tissue was transferred in Dulbecco’s modified Eagle’s medium (DMEM) + Ham’s F12 medium on ice and processed either for immunohistochemistry or for primary cell culture preparation.

#### Cell culture

Endometrial tissue was minced into small pieces and incubated with collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 10 min, followed by filtration, as previously described (Tulac et al., 2006; Aghajanova et al., 2009). This method produces 95–99% pure stromal fibroblasts. All cells were then cultured in DMEM-F12 without phenol red (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mM l-glutamine (Gibco) and 1% antibiotics–antimycotics (Gibco) up to passages 4–6. Medium was replaced every 3 days. Cells were cultured in Petri dishes coated with fibronectin (Gibco) and collagen (Gibco). To exclude the influence of the serum-derived steroid hormones, for two passages before experiments the cells were grown in culture medium containing 10% charcoal stripped (CS)-FBS (Gibco).

#### Proliferation assay

Proliferation experiments were performed with the cell proliferation ELISA, BrdU colorimetric™ kit (Roche, Basel, Switzerland) according to the manufacturer’s protocol. Co- or Eu-hESC (0.3 × 10^4 cell/well) were grown in 100 μl in CS-FBS containing DMEM-F12 medium for 24 h in the absence of additional growth stimuli. In case of inhibitory treatments, specific ROCK (Y-27632, 10 μM; Sigma-Aldrich) or MAPK (U0126, 20 μM; Promega, Seattle, WA, USA)
The absorbance in 100 mCi of [3H]thymidine was measured after an additional 24 h. The substrate turnover was quantified using a microplate reader. The absorbance values represent the rate of DNA synthesis and correspond to the number of proliferating cells. These values were normalized to those measured in the respective experimental controls where absorbance was set to 100%.

### Migration assay

Cells were analysed for migration using a Boyden chamber assay with polycarbonate membranes. An equal number of cells (5 x 10⁴) re-suspended in complete cell media were plated to the top fibronectin–collagen-coated chambers (8 μm-pores; BD Biosciences, MA, USA) and allowed to migrate for 12 h. The cells on the underside of the membrane were fixed, stained with 4(6-diamidino-2-phenylindole) and counted (five random fields/membrane) by two independent investigators.

### siRNA knockdowns

Using siLentFect™ (Bio-Rad Laboratories, Hercules, CA, USA) transfection reagent, 10μM ROCKII siGENOME SMART pool (Thermo Scientific Dharmacon, Lafayette, CO, USA) and siCONTROL non-targeting (Thermo Scientific Dharmacon) or 10 nM Raf-1 (Invitrogen, CA, USA; Cat.No: VHS40462) and low-density GCs Duplex siCONTROL non-targeting (Invitrogen; Cat.No: 12935-200) were transfected according to the manufacturer’s protocol. The cells were lysed and analysed 48 h after transfection. B-Raf knockdown was performed with Accell siRNA system using 100 nM oligos (Thermo Scientific Dharmacon; Accell SMART pool siRNA A-003460-20) or Accell non-targeting siRNA (Thermo Scientific Dharmacon; D-001910-01-05) for 72 h, according to the manufacturer’s protocol.

### Cell lysate preparation and western blot analysis

The hESC were harvested, counted and lysed in ice-cold Frakkelton buffer ([110 mM Tris–HCl, pH 7.05; 50 mM NaCl; 30 mM sodium pyrophosphate; 50 mM NaF; 1% Triton X-100; 100 μM Na₃VO₄; 1 tablet of protease inhibitor cocktail (Roche); 1 mM phenylmethylsulphonyl fluoride]). Insoluble material was removed by centrifuging the samples at 20 000 g, 20 min, at 4°C. The cytosolic and nuclear cell lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Chemical Co., Colfax, CA, USA) according to the manufacturer’s protocol. The protein concentrations for total cell lysates, cytosolic or nuclear extracts were determined using the bichinonic acid protein assay method (Thermo Scientific; IL, USA). Twenty micrograms of normalized samples were immunoblotted as previously described (Rubio et al., 2006) and probed with the following primary antibodies: ROCKII, Raf-1, cyclinD1, p27, pERK, ERK, TOPOII-α-tubulin (Sigma-Aldrich) and stomatin [(clone GARP50—a kind gift of R. Prohaska (Hiebl-Dirschmied et al., 1991)] prior to incubation with peroxidase-conjugated secondary antibodies (Pierce Chemical Co.). Bound antibodies were detected by enhanced chemiluminescence plus western blotting detection system (Amersham Pharmacia Biotech, Inc., NJ, USA) and exposed to X-ray films (GE Health Care, UK). Western blots were scanned by hp Scanjet and quantified by ImageJ free web program (http://rsweb.nih.gov/sj/).

### ROCKII activity assay

ROCKII kinase activity was analysed by an enzyme immunoassay, using Cell Biolabs’ 96-well ROCKII activity assay kit (Cell Biolabs, INC., CA, USA). Experiments were performed according to the manufacturer’s protocol, using 10 μl of protein lysate. The total starting protein concentration for every sample was 1 mg/ml.
B-Raf activity assay

B-Raf kinase activity was determined by B-Raf kinase assay kit (Milipore, Temecula, CA, USA) according to the manufacturer’s protocol. The method is designed to measure B-Raf-dependent phosphotransferase activity in a kinase reaction using recombinant inactive MAP kinase/ERK kinase-1 (MEK1) as a B-Raf substrate followed by immunoblotting and chemiluminescence detection. Total cell lysates isolated as described above with 10 μg protein per sample were used. The B-Raf activity from Co-hESC was compared with those of Eu-hESC before and 48 h after their transfection with either control siRNA or with specific Raf-1 siRNA.

Statistical analysis

Data were subjected to statistical analysis using unpaired Student’s t-test.

Results

Cell proliferation and protein expression in hESC from women without and with endometriosis

Cell proliferation in the presence of serum derived growth factor stimulation and absence of sex steroid hormones in Co- and Eu-hESC was measured by BrdU incorporation. The results in Fig. 1A show that proliferation rate of Eu-hESC was 54% (P < 0.05) higher compared with respective controls. In order to better characterize our primary cell system, we first analysed the levels of expression of two Raf isoforms (Raf-1 and B-Raf), which are the potential downstream effectors of Ras in the Ras/Raf/MAPK signalling cascade. Statistical analysis of western blots for Raf-1 in all primary cultures (Table 1) showed down-regulation of Raf-1 expression in Eu-hESC, compared with Co-hESC (Fig. 1B and C, left panel and Supplementary data, Fig. S1). Raf-1 expression levels obtained from Eu-hESC were 47% (P < 0.05) lower than those from Co-hESC (Fig. 1C, left panel). No difference in B-Raf levels between the cell types was observed (Fig. 1B and data not shown). Further, we determined expression levels of panERK, pERK, ROCKII, cyclinD1 and p27 in both cell types. The comparison of the mean values of panERK, cyclinD1 and ROCKII expression levels obtained from Eu-hESC with those obtained from Co-hESC showed no difference between cell types (Fig. 1B and C, left panel). Mean p27 expression levels and ERK activation levels obtained from Eu-hESC were 61% (P < 0.05) and 83% (P < 0.05) higher than those from respective Co-hESC (Fig. 1C, left panel and Supplementary data, Fig. S1). This data confirmed previous observations that Eu-hESC

Figure 1  Cell proliferation and expression analysis in primary Co- and Eu-hESC. (A) Average values of five independent experiments of the percentage of proliferating cells analysed in cell proliferation ELISA BrdU assay are shown. Levels were normalized to Co-hESC, where the percentage of proliferating cells was set to 100%; *P < 0.05. (B) Western blot analysis performed on Co- and Eu-hESC prepared from individual patient tissue samples (patient ID numbers are given on the top of the gel). The levels of ROCKII, B-Raf, the phosphorylation of ERK, panERK, cyclinD1, p27, Raf-1 and α-tubulin (loading control) were determined, TCL, total cell lysates. (C) Graphical representation (left panel) of averaged levels (mean value ± SD) of ROCKII, cyclinD1, p27 and Raf-1 normalized by α-tubulin and obtained by western blot analysis of all hESC used in the study (n = 16 for Eu-hESC and n = 14 for Co-hESC) as described in (B). The data are shown as percent of every protein in Eu-hESC group (mean value ± SD) relative to those in Co-hESC group (set to 100%); *P < 0.05. Graphical representation of immunoblot analysis of pERK normalized by total levels of panERK and α-tubulin for all patient samples used in the study is shown on the right. Data are expressed as protein levels in percent (mean value for every group ± SD) relative to their normalized levels (set to 100%) in Co-hESC group; *P < 0.05.
Ras/Raf/MAPK and Rho/ROCKII signalling pathways are involved in the regulation of cell proliferation of Co- and Eu-hESC

Given the role of Ras/Raf/MAPK and RhoA/ROCKII in the regulation of cell proliferation, we hypothesized that the basal cell proliferation in Co- and Eu-hESC is under the control of both signalling pathways. Therefore, we analysed the effects of the MAPK (U0126, 20 μM) and ROCK (Y-27632, 10 μM) inhibitors on Co- and Eu-hESC proliferation 24 h after their administration. Both inhibitors caused a significant decrease in cell proliferation in Co- and Eu-hESC (Fig. 2). Inhibition of MAPK reduced cell proliferation to 26% in Co-hESC (P < 0.001) and to 24% in Eu-hESC (P < 0.001) of respective control levels. Incubation with 10 μM of ROCK inhibitor (Y-27632) reduced cell proliferation in Co-hESC to 63% (P < 0.001) and in Eu-hESC to 60% (P < 0.001) compared with respective non-treated controls.

RhoA/ROCKII signalling and cell proliferation in Co- and Eu-hESC

To rule out possible ROCKII independent effects of the Y-27632, we analysed the influence of ROCKII siRNA knockdown on cell proliferation in our in vitro cell system. Knockdown of ROCKII in Co-hESC via siRNA to 38% (P < 0.0001) was able to reduce cell proliferation to 48% (P < 0.005) versus controls (Fig. 3A and B). In these cells, ROCKII knockdown was linked to down-regulation of cyclinD1 and p27 protein levels to 38% (P < 0.0001) and 69% (P < 0.01), respectively (Fig. 3A). Reduction of the levels of these two proteins was also observed in Eu-hESC, where 78% (P < 0.0001) ROCKII knockdown was achieved. Here, the effect of knockdown on cell proliferation was slightly stronger (69% down-regulation, P < 0.005 of control levels) compared with ROCKII deficient Co-hESC. However, ROCKII knockdown did not influence the phosphorylation of ERK in both cell types (Fig. 3A and D), suggesting that RhoA/ROCKII affects cell proliferation by a mechanism independent of ERK activation. Since, ROCKII is a known regulator of cellular mobility, we further analysed the effects of ROCKII knockdown on hESC migration. Knockdown of ROCKII caused significant down-regulation of hESC migration. In Co-ROCKII knockdown cells, migration rate was reduced to 35% (P < 0.05) and in Eu-ROCKII knockdown cells to 43% (P < 0.001) compared with respective siRNA controls (Fig. 3C, left panel).

Raf-1 is dispensable for Ras/Raf/MAPK dependent regulation of cell proliferation in hESC

Raf-1 has been shown to be involved in the regulation of cell proliferation as a part of Ras/Raf/MAPK signalling cascade in different cell types (Buitrago et al., 2003; Lefevre et al., 2003). To determine whether and how Raf-1 is involved in the regulation of hESC cell proliferation, we analysed the influence of Raf-1 knockdown in our in vitro cell system. Knockdown of Raf-1 to 20% in Co-hESC (P < 0.0001) and to 6% in Eu-hESC (P < 0.0001), respectively, caused a significant increase in p27 protein levels. Particularly, in Co-hESC, p27 levels were 2.7-fold higher (P < 0.0001) and in Eu-hESC 4.6-fold (P < 0.0001) higher compared with respective siRNA controls (Fig. 4A). Analysis of the subcellular distribution of the p27 protein in both cell types showed that Raf-1 negatively regulates
Figure 3 Effects of ROCKII siRNA knockdown in Co- and Eu-hESC. (A) Total cell lysates (TCL) from Co- and Eu-hESC 48 h after their transfection with ROCKII siRNA or control siRNA were analysed for ROCKII, pERK, panERK, cyclinD1, p27 and α-tubulin expression. The levels for every protein were normalized by total α-tubulin. Representative blots from biological triplicates (left panel) and graphical representation of ROCKII, cyclinD1 and p27 (right panel) are shown as protein levels in percent (mean from five independent experiments ± SD) relative to their normalized levels (set to 100%) in Co-hESC cells transfected with control siRNA; *P < 0.01 and **P < 0.005. (B) Effects of ROCKII knockdown on cell proliferation in Co- and Eu-hESC are shown. Average values of biological triplicates (mean value ± SD) are presented as percentage of proliferating cells measured by ELISA BrdU assay and relative to Co-hESC cells transfected with control siRNA (mean value set to 100%). The P-values for every compared averaged value are indicated on the top. (C) Effects of ROCKII knockdown on cell migration in Co- and Eu-hESC are shown. Average values of biological triplicates are expressed as percentage of migrating cells relative to Co-hESC transfected with control siRNA (mean value set to 100%). *P < 0.05 and **P < 0.005. (D) Western blot analysis (left panel) and graphical representation (right-panel) of cytoplasmic (CE) and nuclear (NE) levels of expression of p27, cyclinD1 and pERK proteins 48 h after ROCKII siRNA knockdown in Eu-hESC are shown. Representative blots from three independent experiments are given. TOPOII-β was used as nuclear-specific loading control and β-actin as loading control in the cytosolic fraction. Protein levels are given in percent (mean from three independent experiments ± SD) relative to their normalized levels (mean value set to 100%) in control siRNA transfected Co-hESC; *P < 0.05 and **P < 0.005.
p27 levels only in the cytoplasm and not in the nuclear cell compartment (Fig. 4B). The down-regulation of Raf-1 did not affect the activity of ERK and the level of cyclinD1 (Fig. 4A), suggesting that it is not an effector of Ras in the Ras/Raf/MAPK signalling cascade. In addition, the loss of Raf-1 did not cause significant changes in Co- and Eu-hESC proliferation (Fig. 4C).
Raf-1 is a negative regulator of ROCKII activation in Co- and Eu-hESC

Recently, the physical interaction of ROCKII and Raf-1 was shown to negatively regulate ROCKII activity in mouse keratinocytes and fibroblasts (Ehrenreiter et al., 2005). Based on this finding, we hypothesized that Raf-1 operates as a negative regulator of ROCKII activity in our cell system and that the RhoA/ROCKII signalling pathway could be abnormally regulated in Eu-hESC, where the expression of Raf-1 is significantly lower than that in Co-hESC (Fig. 1). We analysed ROCKII activity in Co- and Eu-hESC 48 h after their transfection either with control siRNA or with specific Raf-1 siRNA. Results presented on Fig. 4D (left panel) show that Co-hESC exhibit lower ROCKII activity than Eu-hESC. In Eu-hESC transfected with control siRNA, ROCKII activity was 1.85-fold (P < 0.005) than in corresponding Co-hESC. Knockdown of Raf-1 was able to strongly activate ROCKII in both cell types. In Co-Raf-1-knockdown cells, ROCKII activity was 2.3-fold (P < 0.001) and in Eu-Raf-1-knockdown cells to 1.9-fold (P < 0.001) higher than in corresponding siRNA controls. In summary, these experimental results support our hypothesis and show that Raf-1 operates as a negative regulator of ROCKII activity in hESC and that in Eu-hESC the RhoA/ROCKII signalling pathway is abnormally activated.

To further investigate whether ROCKII activity is sufficient for p27 regulation, the Co-Raf-1-knockdown cells were treated with Y-27632 (10 μM) for 24 h. The data presented in Supplementary data, Fig. S2 show that ROCKII inhibition in cells transfected with control siRNA leads to down-regulation of p27 protein levels. Y-27632 administration in Raf-1-knockdown cells abolished the effect of ROCKII on p27. No effect of ROCKII inhibition on cyclinD1 levels was observed. Therefore, in hESC, ROCKII activity alone is sufficient for p27 (but not for cyclinD1) regulation.

Raf-1 knockdown affects migration of Co- and Eu-hESC

Since, Raf-1 was dispensable for cell proliferation control in Co- and Eu-hESC, we further investigated whether it can affect hESC mobility. The migration rate of Eu-hESC transfected with control siRNA was 75% higher compared (P < 0.001) with respective Co-hESC (Fig. 4D, right panel). Knockdown of Raf-1 caused a significant down-regulation of cell migration in both cell types. In Co-Raf-1-knockdown cells, the migration rate was reduced to 49% (P < 0.01) and in Eu-Raf-1-knockdown cells to 66% (P < 0.001) compared with respective siRNA controls. These data show that Raf-1 operates as a regulator of hESC migration.

Further, we analysed the effects of Raf-1 knockdown on B-Raf kinase activity in hESC. The results presented in Fig. 5 show that B-Raf activity in Eu-hESC is 2.4-fold (P < 0.0005) higher compared with Co-hESC (compare lane 4 with lane 5 in Fig. 5A, left panel). No differences were observed between the same type of cells transfected either with Raf-1 siRNA or with control siRNA (compare lane 6 with lane 7 for Eu-hESC and lane 8 with lane 9 for Co-hESC on Fig. 5A, left panel). These experimental data confirm our observations that Raf-1 is not involved in Ras/Raf/MAPK signalling and show that Eu-hESC exhibit a significantly higher basal B-Raf kinase activity compared with Co-hESC.

B-Raf regulates cell proliferation in hESC as part of the Ras/Raf/MAPK signalling pathway

To assess the role of B-Raf in the regulation of hESC proliferation, we performed knockdown experiments in Co- and Eu-hESC using a specific B-Raf siRNA. Western blot analysis showed that the down-regulation of cellular B-Raf protein levels to ~33% (P < 0.001) in both cell types caused significant down-regulation of cyclinD1 levels.
to 55% for Co- (P < 0.001) and 62% (P < 0.01) for Eu-hESC, respectively (Fig. 6A and B). This effect was accompanied by significant changes in ERK activation (Fig. 6A and C). In Co-hESC, B-Raf knockdown results in 37% (P < 0.05) down-regulation of ERK phosphorylation and in Eu-hESC 55% (P < 0.001) down-regulation, when compared with respective controls (Fig. 6A and C). The levels of p27 protein did not change in both cell types under B-Raf knockdown (Fig. 6A). This data show that B-Raf acts as an up-stream regulator of ERK in Ras/Raf/MAPK signalling, thereby controlling the cellular levels of cyclinD1 protein. Further, we analysed the effects of B-Raf knockdown on hESC proliferation. In B-Raf-knockdown cells, the proliferation rate was reduced to 28% (P < 0.001) in Co-hESC and by 50% (P < 0.05) in Eu-hESC compared with respective iRNA controls (Fig. 6D).

**Discussion**

Our data on primary cultures of hESCs suggest a novel understanding of molecular mechanisms underlying their basal regulation of proliferation and migration. We demonstrate that in hESC, the control of cell proliferation is associated with the regulation of cell migration. For the first time, we show that the RhoA/ROCKII signalling pathway operates as a dual regulator of basal proliferation and migration in hESC. Moreover, we provide evidence that the same molecular mechanisms operate in hESC isolated from normal endometrium as well as in eutopic endometrium obtained from patients with endometriosis. The differences between Eu- and Co-hESC are due to abnormal activation of Ras/Raf/MAPK and RhoA/ROCKII signalling pathways in diseased cells, which probably ensure their different sensitivity to specific stimuli.

Inhibition of the downstream RhoA effector ROCKII was previously shown to suppress cell proliferation in different cell types (Kamiyama et al., 2003; Zhao and Rivkees, 2003; He et al., 2005). We found that in hESC, ROCKII regulates basal cell proliferation via regulation of cyclinD1 levels, by a mechanism independent of Ras/Raf/MAPK activation. Direct ROCKII-dependent and MAPK-independent mechanism of cyclinD1 regulation was previously proposed in NIH3T3 cells (Croft and Olson, 2006). The hyperactivation of ROCKII under Raf-1 knockdown was dispensable for cell proliferation control in hESC. This observation agrees with previously published data from others.
showing that the activated RhoA/ROCKII alone is not able to promote cyclinD1 induction and G1-progression in serum-starved fibroblasts (Gjoerup et al., 1998; Welsh et al., 2001). Therefore, only significant reduction of ROCKII protein and activity has a function in hESC proliferation control. RhoA/ROCKII is an important regulator of normal cytoskeleton organization (Hall, 1998), the integrity of which is necessary for cyclinD1 induction in the G1-phase as well as cell-cycle progression (Welsh et al., 2001). This could explain why the loss of ROCKII leads to strong down-regulation of cell proliferation. Several studies (Balmanno and Cook, 1999; Roovers and Assoian, 2003; Roovers et al., 2003) show that RhoA/ROCKII functions as the major adhesion-dependent regulator of cyclinD1 expression. Thus, the duration of the G1-phase is responsive to the formation of actin stress fibres and the induction of intracellular tension. Although we clearly show that Raf-1/RhoA/ROCKII signalling pathway is an important regulator of cell proliferation in hESC, we cannot directly link the enhanced basal cell proliferation capacity of Eu-hESC to the abnormal activation of RhoA/ROCKII signalling pathway. We propose that the main function of RhoA/ROCKII is to ensure cytoskeleton environment needed for basal cell proliferation.

We show that in the absence of specific stimulus, Eu-hESC display abnormal activation of Raf-1/RhoA/ROCKII signalling pathway, high levels of ERK activation, but not significantly different cyclinD1 levels compared with Co-hESC. Tightly coordinated interactions between Rho-GTPases facilitate cell-cycle progression by regulating the expression of cyclinD1 and assembly of cyclinD/cdk complexes under growth factor stimulation (Burbelo et al., 2004). Rac and Cdc42 also induce cyclinD1 expression independent of ERK and involve the NF-kB signalling pathway (Westwick et al., 1997; Joyce et al., 1999; Burbelo et al., 2004). RhoA activation suppresses Rac/Cdc42-dependent cyclinD1 induction through the ROCKII downstream effector-LIMK (Roovers et al., 2003). In addition, cyclinD1 was proposed to function as a feedback-regulator of ROCKII expression and activation (Li et al., 2006). Collectively, these studies show that the regulation of cellular cyclinD1 levels by Rho-GTPases under specific growth factor stimulation is a complex multisided and dynamic process strongly depending on the timing of activation, interaction and feedback regulation of several proteins. Therefore, although Eu-hESC exhibit abnormally activated ROCKII and ERK, this does not automatically pertain to the levels of cyclinD1. However, these features of Eu-hESC might ensure their higher sensitivity to specific stimuli, compared with Co-hESC. In accordance, Murk et al. showed that oestrogen rapidly stimulates ERK in Eu-hESC but not in Co-hESC (Murk et al., 2008). Oestrogen stimulation was additionally shown to enhance hESC cell mobility, which is controlled by the rapid activation of ERK (Gentilini et al., 2007) or RhoA/ROCKII (Flamini et al., 2009). The challenge for the future will be to determine whether and how the differences in Rac, Cdc42 and RhoA/ROCKII activities under specific growth factor stimulation could account for the timing of G1 cell cycle events in Co- and Eu-hESC.
In smooth muscle cells (Sawada et al., 2000) and hepatocytes (Iwamoto et al., 2000), the inhibition of Rho-associated kinases was reported to up-regulate p27 levels and to suppress G1 to S phase progression. Others, however, have not observed a role for Rho-associated kinases in the regulation of p27 (Sahai et al., 2001). Our data propose that ROCKII controls p27 protein levels in hESC. ROCKII inactivation leads to reduction of p27 in the cytoplasm but not in the nucleus, where it is involved in the regulation of cell proliferation. This is supported by the observation that the hyperactivation of ROCKII after Raf-1 knockdown was dispensable for proliferation control in our in vitro cell system. The loss of Raf-1 did not have an effect on cellular cyclinD1, ERK and pERK levels. Differential expression of Raf-1 during the establishment of endometriosis was previously reported (Wu et al., 2006); however, its function has not been elucidated. Here, we show that in hESC, Raf-1 operates as regulator of cell migration and is not involved in cell proliferation control as part of the Ras/Raf/MAPK signalling cascade. For the first time, we demonstrate that Raf-1 protein levels are lower in Eu-hESC cells, compared with Co-hESC, accompanied by gradual up-regulation of ROCKII activity and significant up-regulation of cytosolic p27 levels. Knockdown of Raf-1 leads to significant up-regulation of ROCKII activity in endometrial cells and therefore, Raf-1 acts as a negative regulator of ROCKII. In addition, as part of the RhoA/ROCKII signalling pathway, Raf-1 negatively regulates cytosolic p27 protein, but does not affect its levels in the nucleus. This could partially explain why Raf-1 knockdown did not have an effect on cell proliferation and additionally suggests that Raf-1 could be important for p27 cell-cycle-independent function. A growing body of literature now suggests that p27 is linked to the regulation of apoptosis (Eymin et al., 1999; Philipp-Staheli et al., 2001) and could regulate cell motility. For example, McAllister et al. (2003) show that a cytoplasmic pool of p27 stimulates Rac-dependent migration of HepG2 cells and embryonic fibroblasts. p27 was also reported to regulate the RhoA activity in the cell (Bess et al., 2004). Our data show that in both cell types, p27 is under the control of the Raf-1/ROCKII signalling pathway. This pathway is up-regulated in Eu-hESC due to lower levels of Raf-1 expression. This yields to an enhanced migratory potential associated with higher levels of p27 compared with Co-hESC. Independent of the fact that our data did not elucidate the function of p27 as regulator of cell migration in endometriosis, this possibility still exists, and it will be an intriguing issue for the future to address the role of Raf-1/RhoA/ROCKII-dependent p27 regulation in establishment of the disease.

Here, we identify B-Raf as an upstream regulator of MAPK pathway in hESC. We show that the specific siRNA B-Raf knockdown leads to significant down-regulation of ERK activation, reduction of the cellular cyclinD1 protein and significant down-regulation of cell proliferation compared with respective siRNA controls. B-Raf was already shown to be the main activator of the MAPK pathway in several cell types (Catling et al., 1994; Traverse and Cohen, 1994). Our experimental data show that the Co-hESC and Eu-hESC express similar levels of B-Raf but differ with respect to the levels of B-Raf activation. The Eu-hESC display higher B-Raf activity compared with Co-hESC and, therefore, abnormally activated Ras/B-Raf/MAPK signalling results in abnormal ERK activation and higher proliferation rate in comparison with Co-hESC.

In conclusion, we herein provide evidence for a cooperative regulation of proliferation and migration at the level of ROCKII in hESC, harvested mainly from proliferative phase endometrium of patients and controls. ROCKII functions as an integration point between proliferation and migration and its expression level seems to regulate specific cell behaviour. When ROCKII is lost, hESC are not able to proliferate most probably due to changes in basal cytoskeleton organization and deregulation of cell-cycle protein cyclinD1 (Fig. 7). Indirect evidence that such cytoskeleton reorganization takes place in ROCKII-deficient cells is driven from the observation that they show significant reduction of their migration rate. However, ROCKII hyperactivation results in dissociation of migration from proliferation control, leading to down-regulation of migration on the background of unchanged proliferation in the absence of specific stimuli. hESC of patients with endometriosis exhibit higher Raf-1/RhoA/ROCKII and B-Raf/MAPK signalling activation and higher ROCKII and B-Raf activities compared with Co-hESC. The higher ERK activation would be responsible for elevated proliferation ability of diseased over control cells (Fig. 7). Abnormally activated Raf-1/RhoA/ROCKII in Eu-hESC ensures their increased migratory potential over Co-hESC. Therefore, the hESC from patients with endometriosis would behave differently under the same stimuli and cell environment compared with controls. These mechanisms of cellular regulation could explain the incomplete transitioning of the diseased endometrium and its enhanced proliferative and migratory phenotype.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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