Resveratrol enhances decidualization of human endometrial stromal cells

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Abstract

The differentiation of endometrial stromal cells (ESC), named decidualization, is essential to regulate trophoblast invasion and to support pregnancy establishment and progression. Decidualization follows ESC proliferation and it has been described that cell cycle arrest contributes to a proper decidualization. Interestingly, resveratrol, a natural compound derived from grapes with antioxidant properties, has been widely studied in relation to endometrial health. However, little is known about the effect of resveratrol supplementation during decidualization. Therefore, in this study we evaluate the effect of resveratrol supplementation during decidualization. We used primary and immortalized human ESC and we decidualized them *in vitro* with a decidualization cocktail containing medroxyprogesterone acetate, estradiol and 8-Bromo-cyclic AMP. Pre-decidualized cells were further treated with the decidualization cocktail supplemented with resveratrol. Our results show that resveratrol supplementation increased, in a dose-dependent manner, the expression levels of prolactin and *IGFBP1* (RT-PCR and ELISA), indicating an enhanced *in vitro* decidualization of human ESC. This enhanced decidualization was accompanied by a decrease in cell proliferation (crystal violet and CellTiter proliferation assay) and by changes in the mRNA levels of key cell cycle regulators (RT-PCR). Furthermore, resveratrol supplementation seemed to enhance decidualization by reinforcing the effect of the decidualization cocktail. We believe that resveratrol could be an effective supplementation to reinforce hormone action during human ESC decidualization and that further insights into resveratrol action and its interaction with estradiol and progesterone signaling pathways could facilitate the identification of new therapeutic strategies for the improvement of women's health.

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Introduction

During the menstrual cycle, the endometrium undergoes cyclic rounds of cell proliferation, differentiation and death. After ovulation, during the secretory phase of the menstrual cycle, the differentiation of human ESC takes place. This differentiation process is known as decidualization and is a tightly regulated process. Decidualization comprises of a profound change in cell morphology and function that is accompanied by a complete reprogramming of gene and protein expression (Ramathal *et al.* 2010). This change in cell identity is critical to ensure proper fetomaternal interactions and to guide placentation, ensuring a healthy pregnancy (Strowitzki *et al.* 2006, Gellersen & Brosens 2014).

It has been widely described that cell cycle arrest is an important event related to cell differentiation (Reichert & Eick 1999, Myster & Duronio 2000, Kwon *et al.* 2016, Ruijtenberg & van den Heuvel 2016, Soufi & Dalton 2016). Cell cycle progression is tightly regulated by the coordinated action of cyclin-dependent kinases (CDK). CDK activity is regulated by cyclins and CDK inhibitors (CDKI) that are expressed in a timely manner during the

cell cycle. In relation to endometrial function, it has been demonstrated that the expression of several cell cycle regulators is modified during decidualization and that ESC exit the cell cycle before differentiating (Takano *et al.* 2007, Logan *et al.* 2012). In particular, previous findings have determined that ESC are arrested at the G0/G1 checkpoint during decidualization (Wang *et al.* 2018). The activation and crosstalk of progesterone (P) and cyclic AMP (cAMP) signaling pathways regulate the changes in cell cycle progression (Gellersen & Brosens 2003). Furthermore, P and cAMP are key to drive the whole protein and gene expression reprogramming of ESC during decidualization (Gellersen & Brosens 2014).

Defective decidualization has been associated with impaired pregnancy establishment and progression (Strowitzki *et al.* 2006). For example, it has been studied that impaired decidualization could have an important role during the onset of preeclampsia, a placenta-related pathology (Garrido-Gomez *et al.* 2017). Moreover, it has been demonstrated that ESC derived from women with endometriosis, a benign disorder defined by the presence of endometrial tissue outside the uterine cavity, have reduced expression of decidualization

markers compared to ESC derived from women without endometriosis (Klemmt *et al.* 2006, Aghajanova *et al.* 2009).

Interestingly, resveratrol (R) (3,4',5-trihydroxy-trans-stilbene), a natural polyphenolic compound derived from some fruits and vegetables, has been widely studied in relation to reproductive health. Several authors have described the beneficial properties of R in relation to the inhibition of cell proliferation and migration and the reduction of oxidative stress and pointed to R as a possible drug for the treatment of endometriosis and preeclampsia (Ricci *et al.* 2013, Zou *et al.* 2014, Hannan *et al.* 2017, Kolahdouz Mohammadi & Arablou 2017). Furthermore, it has been shown that R exerts a positive effect over fertility in relation to ovarian aging in mice, as the long-term oral administration of R improved the number and quality of oocytes, allowing old, female mice to retain more capacity to reproduce (Liu *et al.* 2013, Ortega & Duleba 2015).

Due to the described beneficial effects of R in women's reproductive health, we were interested in evaluating the effect of R supplementation during decidualization. Therefore, the aim of this study was to evaluate the effect of resveratrol supplementation during *in vitro* decidualization of human ESC. Herein, we describe that resveratrol supplementation together with medroxyprogesterone acetate (MPA), estradiol and 8-Bromo-cAMP (8-Br-cAMP) enhances the decidualizing effect of these drugs leading to a higher expression of prolactin (PRL) and Insulin-like growth factor binding protein 1 (IGFBP1). In addition, we describe that R supplementation enhances the inhibition in cell proliferation that the decidualization program requires, probably contributing additionally, this way, to ESC differentiation.

Materials and methods

Ethical approval

This study protocol was approved by the ethical committee (S-239/2005) of the Ruprecht-Karls University of Heidelberg. Biopsies were taken after written informed consent was obtained from all participants.

Cell isolation and culture

Primary cells

Endometrial biopsies from mid-late proliferative phase were obtained from healthy regularly-cycling women (34.4 ± 1.1 years old) without hormonal therapies, no endometrial abnormalities and no endometriosis at diagnostic laparoscopy. Endometrial biopsies were cut into small pieces and digested with collagenase (Gibco) and hyaluronidase (Sigma-Aldrich) at 37°C for 60 to 90 min. To remove epithelial cells, cell suspensions were filtered through a 40 µm mesh. Isolated primary human ESC (HESC) were cultured in a 3:1 mixture of DMEM (Cat Number 21063, Gibco) and MCDB-105 (Cat

Number M6395, Sigma-Aldrich) without phenol red and supplemented with 10% v/v charcoal/dextran treated fetal bovine serum (FBS) (HyClone, GE Healthcare Europe). Cell passages number two to four were used for the experiments described subsequently.

Cell line

Immortalized human ESC (t-HESC) (Krikun *et al.* 2004) were purchased from LGC (ATCC CRL-4003, LGC Standards GmbH, Wesel, Germany) and cultivated in DMEM-F12 (Cat Number D2906, Sigma-Aldrich) without phenol red according to ATCC recommended instructions.

In vitro decidualization and resveratrol supplementation

Cells were trypsinized, counted and plated in 6-well plates (150,000 cells/well for HESC and 100,000 cells/well for t-HESC). After 24–48 h, when cells had reached 80–90% confluency, medium was removed and cells were rinsed with PBS. Then, 2 mL of fresh 2% v/v FBS medium supplemented with a decidualization cocktail (DC) containing 1 µM medroxyprogesterone acetate (MPA), 10 nM estradiol (E) and 0.375 mM 8-Br-cAMP (D, decidualized) or vehicle solutions (ND, non-decidualized) were added to each well. At days 3 and 5 of the decidualization treatment, medium was changed and different doses of resveratrol (0 vehicle treated; 6.25; 12.5; 25; 50 µM) were added to each well. Only cells treated with the DC were supplemented with resveratrol. After 2 and 4 days of resveratrol supplementation (days 5 and 7 of the decidualization protocol), cell supernatants (SN) were collected for the quantification of prolactin levels and cell samples were collected in TRIzol reagent (Invitrogen, Life Technologies GmbH) for total RNA extraction or in RIPA buffer for protein detection by Western blot. For microtubule-associated protein light chain 3 B (LC3B) detection by Western blot, chloroquine (25 µM) or vehicle solution were added to the culture medium 8 h before harvesting the cells. Drugs used are listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Prolactin quantification

Prolactin from cell culture supernatant was quantified at the central laboratory of Heidelberg University Clinic using a double sandwich ELISA assay (Siemens Diagnostics) according to the manufacturer's instruction.

Gene expression assays

Gene expression assays were performed exactly as previously described (Mestre Citrinovitz *et al.* 2019). Primers used are listed in Supplementary Table 1. RT-PCR reactions were run in the Fast Forward 7500 real-time PCR-system (Applied Biosystems, Life Technologies GmbH). RT-PCR results were analyzed according to the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001), and *RPLP0* was used as internal control for normalization of C_t values. Normalized C_t values (ΔC_t) were used for statistical analysis.

Proliferation assays

t-HESC and HESC were trypsinized, counted and plated in 96-well plates at a density of 1000 cells/well. After 24–48 h, medium was removed and 100 μ L of fresh 2% v/v FBS medium containing the DC were added to each well. At days 3 and 5 of the decidualization treatment, medium was changed and cell cultures were supplemented with or without 25 μ M of resveratrol. After 4 days of resveratrol supplementation (day 7 of the decidualization protocol), cell proliferation was evaluated by crystal violet (CV) staining and by CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega Corporation). Proliferation assays were performed according to manufacturer's instructions and exactly as previously described (Feoktistova *et al.* 2016, Mestre Citrinovitz *et al.* 2019).

Western blot analysis for protein detection

LC3B and p62 detection by Western blot was performed exactly as previously described (Mestre Citrinovitz *et al.* 2019). Antibodies used and working dilutions are listed in Supplementary Table 1.

Statistical analysis

Paired Student's *t*-test and one-way ANOVA followed by Dunnett's Multiple Comparison Test were used for statistical analysis. Differences between treatments were considered significant when $P < 0.05$. Statistical analysis was carried out using GraphPad Prim 4.0 (GraphPad Software Inc.).

Results

Resveratrol supplementation enhances HESC decidualization

As a first approach to evaluate the effect of R supplementation during the decidualization of HESC, we measured the expression level of *PRL* and *IGFBP1*, two well-known decidualization markers (Brosens *et al.* 1996), in HESC treated with the DC supplemented with different doses of R. As expected, after 5 and 7 days of DC treatment, *PRL* protein levels and *PRL* and *IGFBP1* mRNA levels were increased in decidualized (D) cells compared to non-decidualized (ND) cells (Supplementary Fig. 1). R supplementation further increased *PRL* protein levels and *PRL* and *IGFBP1* mRNA levels in a dose-dependent manner compared to 0 μ M of R (Fig. 1A and B).

Then, we decided to evaluate the changes in mRNA expression of other secreted factors known to be regulated during decidualization: *IL-8*, a cytokine related to trophoblast invasion, and *VEGFA*, an important proangiogenic factor. The mRNA levels of both genes were increased during decidualization (Supplementary Fig. 1). However, in response to R supplementation *IL-8* mRNA levels were decreased in a dose-dependent manner, while *VEGFA* mRNA levels were slightly diminished only by 50 μ M of R (Fig. 2A).

To move forward in the description of the effect of R supplementation during the decidualization, we decided to evaluate the mRNA levels of *HOXA10* (Qian *et al.* 2005, Godbole *et al.* 2017), *FOXO1* (Kajihara *et al.* 2013) and *NOTCH1* (Afshar *et al.* 2012), three genes tightly related to gene expression regulation

Figure 1 Resveratrol supplementation increases *PRL* and *IGFBP1* expression during *in vitro* decidualization of HESC. HESC were treated with DC for 3 days and then treated with DC plus different concentrations of R for 2 or 4 more days. (A) *PRL* levels in cell culture supernatant ($n = 5$). Data represent mean mU/l $\pm$ s.e.m. (B) *PRL* and *IGFBP1* mRNA expression levels ($n = 5$). Data represent mean fold change compared to 0 μ M R (dotted line, fold change = 1.0) $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to 0 μ M of R). DC: decidualization cocktail; R: resveratrol; light grey: day 5; dark grey: day 7.

Figure 2 Resveratrol supplementation during *in vitro* decidualization of HESC regulates the mRNA expression of several genes associated with decidualization. HESC were treated with DC for 3 days and then treated with DC plus different concentrations of R for 2 or 4 more days. (A) Secreted factors: *IL-8* and *VEGFA* mRNA expression levels ($n=5$). (B) Genes associated to gene expression regulation and cell signaling: *HOXA10*, *FOXO1* and *NOTCH1* mRNA expression levels ($n=5$). Data represent mean fold change compared to 0 μM R $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to 0 μM of R, dotted line, fold change = 1.0). DC: decidualization cocktail; R: resveratrol; light grey: day 5; dark grey: day 7.

and to cell signaling during decidualization (Fig. 2B and Supplementary Fig. 1). *HOXA10* and *NOTCH1* mRNA expression was decreased in response to the DC. In addition, *HOXA10* and *NOTCH1* mRNA levels were further decreased by R supplementation in a dose-dependent manner. In contrast, *FOXO1* mRNA levels were increased during HESC decidualization (Supplementary Fig. 1), and R supplementation had no additional effect (Fig. 2B).

These results indicate that R supplementation reinforces the effect of DC over HESC during decidualization. This increased decidualization was confirmed by the further increased expression of *PRL* and *IGFBP1* upon R supplementation. In addition, *HOXA10* and *NOTCH1* mRNA levels, decreased in response to the DC, were further decreased by R supplementation.

***t*-HESC respond similar to HESC when supplemented with resveratrol during decidualization**

Primary cells isolated from patients' biopsies are an excellent and widely used experimental model to study cellular response to different drugs and to uncover the signaling pathways involved in that response. However, primary cells are a limited source and the use of established cell lines helps to overcome this issue. Therefore, we decided to evaluate if *t*-HESC, a cell line that has a normal respond to decidualization stimulation (Krikun *et al.* 2004), can be used as an experimental model for the study of the effect of R supplementation during decidualization.

To compare both cell types in relation to their response to R supplementation, we treated *t*-HESC as described for HESC and we measured the changes in *PRL* and *IGFBP1* expression levels. We decided to measure only at day 5 of the decidualization treatment, as the effect of R was already evident by that time point in HESC (Fig. 1). After 5 days of decidualization treatment, *PRL* protein as well as *PRL* and *IGFBP1* mRNA levels were increased in D cells compared to ND cells (Supplementary Fig. 2). As seen for HESC, R supplementation in *t*-HESC increased the expression levels of *PRL* protein and *PRL* and *IGFBP1* mRNA in a dose-dependent manner compared to 0 μM of R (Fig. 3A and B).

To further compare the effect of R supplementation over *t*-HESC and HESC decidualization, we evaluated the expression levels of *HOXA10*, *FOXO1* and *NOTCH1* in *t*-HESC (Fig. 3C and Supplementary Fig. 2). As seen for HESC, the expression levels of *HOXA10* and *NOTCH1* in *t*-HESC were decreased in response to the DC. However, *HOXA10* and *NOTCH1* mRNA levels were not further modified by R supplementation. Also, as seen for HESC, mRNA levels of *FOXO1* were increased by the DC in *t*-HESC and were not further modified by R supplementation.

These results indicate that R supplementation reinforces the effect of DC over *t*-HESC during decidualization, confirmed by the increased expression of *PRL* and *IGFBP1* upon R supplementation. These results additionally indicate that *t*-HESC are a useful experimental model to evaluate the effect of R supplementation during human endometrial stromal cells differentiation; however,

Figure 3 Resveratrol supplementation increases *PRL* and *IGFBP1* expression during *in vitro* decidualization of t-HESC. t-HESC were treated with DC for 3 days and then treated with DC plus different concentrations of R for 2 more days. (A) PRL levels in cell culture supernatant ($n=4$). Data represent mean $\text{mU/l} \pm \text{S.E.M.}$. (B) *PRL* and *IGFBP1* mRNA expression levels ($n=4$). (C) Genes associated to gene expression regulation and cell signaling: *HOXA10*, *FOXO1* and *NOTCH1* mRNA expression levels ($n=4$). For PCR (B and C), data represent mean fold change compared to $0 \mu\text{M}$ R (dotted line, fold change = 1.0) $\pm \text{S.E.M.}$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to $0 \mu\text{M}$ of R). DC: decidualization cocktail; R: resveratrol.

caution should be taken when evaluating the signaling pathways involved in this decidualization reinforcement.

Resveratrol supplementation reduces cell proliferation during t-HESC and HESC decidualization

As cell differentiation is tightly related to cell proliferation and because R has been described to inhibit cell proliferation in a wide variety of cell types (Cucciolla *et al.* 2007, Delmas *et al.* 2011, Ko *et al.* 2017, Kolahdouz Mohammadi & Arablou 2017), we decided to evaluate if the enhancing effect of R supplementation over t-HESC decidualization could be related to changes in the proliferation capacity of human ESC. Therefore, we evaluated the mRNA levels of *Ki67*, a well-known proliferation marker. The mRNA levels of *Ki67* were decreased during t-HESC decidualization, and *Ki67* mRNA expression was further decreased, in a dose-dependent manner, by R supplementation (Fig. 4A and Supplementary Fig. 3).

To quantify the reduction in t-HESC proliferation, we performed the CV staining and CellTiter proliferation assays. For these assays we tested the $25 \mu\text{M}$ dose of R, as it was the lowest dose to achieve a significant reduction of *Ki67* expression (Fig. 4A) and, also, seemed to be the most effective dose to achieve a positive effect during decidualization. Both proliferation assays showed that the $25 \mu\text{M}$ dose of R was effective to reduce t-HESC proliferation by 14 and 15%, respectively (Fig. 4C and D).

Additionally, we tested if $25 \mu\text{M}$ of R affected the proliferation of HESC during decidualization. We found that *Ki67* mRNA levels were decreased during HESC decidualization; however, *Ki67* mRNA expression was not significantly decreased by R supplementation (Fig. 4B and Supplementary Fig. 3). Moreover, we performed for HESC the CV staining and CellTiter proliferation assays. Both proliferation assays showed that $25 \mu\text{M}$ of R reduced HESC proliferation by 5% compared to $0 \mu\text{M}$ R; however, this reduction was only significant for the CV proliferation assay (Fig. 4E and F).

These results indicate that the increased decidualization resulting from R supplementation is accompanied by a decreased cell proliferation, suggesting a possible functional connection between proliferation, decidualization and the effect of R supplementation.

Resveratrol supplementation during t-HESC and HESC decidualization regulates the mRNA expression of several genes associated with cell cycle progression

To understand better how cell proliferation was affected by R supplementation during t-HESC and HESC decidualization, we evaluated the changes in mRNA expression of key genes involved in cell cycle regulation. In particular, we evaluated the expression of *CDC2* (*CDK1*), *CDK2* and *CDK4*, cyclin A1 (*CCNA1*), B1 (*CCNB1*) and D1 (*CCND1*) and *p21* and *p53* (Figs 5, 6 and Supplementary Fig. 3).

Figure 4 Resveratrol supplementation during *in vitro* decidualization of t-HESC and HESC decreases cell proliferation. (A and B) t-HESC and HESC were treated with DC for 3 days and then treated with DC plus different concentrations of R for 2 more days. Graph shows *KI67* mRNA expression levels (t-HESC: A; $n=4$; HESC: B $n=5$). Data represent mean fold change compared to 0 μM R $\pm$ S.E.M. ** $P < 0.01$; *** $P < 0.001$ (compared to 0 μM of R, dotted line, fold change = 1.0). For the proliferation assays, t-HESC and HESC were treated with DC for 3 days and then treated with DC plus 25 μM of R or vehicle for 4 more days. (C) t-HESC, Crystal Violet proliferation assay ($n=5$). (D) t-HESC, CellTiter 96 Aqueous One Solution Cell Proliferation assay ($n=5$). (E) HESC, Crystal Violet proliferation assay ($n=4$). (F) HESC, CellTiter 96 Aqueous One Solution Cell Proliferation assay ($n=4$). Values inside the bar graph represent the percentage of proliferation. Data represent mean absorbance $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$. DC: decidualization cocktail; R: resveratrol; light grey: day 5; dark grey: day 7; dotted bars: t-HESC; empty bars: HESC.

In contrast to what we observed for *KI67*, the mRNA levels of *CCNA1* were increased during t-HESC decidualization and *CCNA1* expression showed a tendency to be slightly further increased by R supplementation (50 μM). The mRNA levels of *CCNB1* and *CCND1* and of *CDC2*, *CDK2* and *CDK4* were decreased during t-HESC decidualization, and their expression was further decreased by R supplementation in a dose-dependent manner (Fig. 5 and Supplementary Fig. 3), as seen for *KI67*. In relation to cell cycle inhibitors, mRNA levels of *p53* did not change during t-HESC decidualization; however, R supplementation decreased *p53* mRNA levels. The mRNA levels of *p21*, a *p53*-dependent gene, were slightly decreased during t-HESC decidualization and had no further changes with R supplementation (Fig. 5 and Supplementary Fig. 3).

During HESC decidualization (Fig. 6 and Supplementary Fig. 3), the mRNA levels of *CCNA1* were decreased and R supplementation had no further effect. The mRNA levels of *CCNB1*, *CCND1*, *CDC2* and *CDK2* were decreased during HESC decidualization, and their expression was further decreased by R supplementation. The expression of *CDK4* was not modified during HESC decidualization; however, it was decreased by R supplementation (Fig. 6 and Supplementary Fig. 3). Finally, the mRNA levels of *p53* were not changed during HESC decidualization, while R supplementation decreased *p53* mRNA levels. The mRNA levels of *p21* were highly increased during t-HESC decidualization and were decreased upon R supplementation.

These results suggest that R supplementation inhibits cell proliferation, mainly by decreasing the expression of positive regulators of cell cycle progression during t-HESC and HESC decidualization.

Resveratrol supplementation during t-HESC decidualization does not affect autophagic flux

Finally, we have recently shown that macroautophagy (herein referred to as autophagy), a highly conserved degradation pathway, is increased during t-HESC decidualization (Mestre Citrinovitz *et al.* 2019). Furthermore, the transfection of small interference RNA (siRNA) against autophagy related (ATG) proteins 7 and 5, key proteins involved in the autophagy pathway, led to the impairment of decidualization, highlighting the importance of these proteins and of the autophagy pathway for the proper decidualization of human ESC.

Interestingly, it has been described that R is capable of activating autophagic flux (Hasima & Ozpolat 2014). Therefore, we evaluated whether R supplementation was able to increase autophagic flux during t-HESC decidualization. We measured the protein levels of LC3BII, the lipidated and autophagosome-associated form of LC3B, in a system where autophagic flux was blocked with chloroquine (CQ) and of p62 in a CQ-free system. Both proteins are degraded when autophagy is active and they are commonly used to evaluate changes in the autophagic flux (Klionsky *et al.* 2016). In this study, we found no differences in the protein levels of

Figure 5 Resveratrol supplementation during *in vitro* decidualization of t-HESC regulates mRNA expression of several genes associated with cell cycle progression. t-HESC were treated with DC for 3 days and then treated with DC plus different concentrations of R for 2 more days. Graphs show (A) *CDC2*, *CDK2*, *CDK4*; (B) *CCNA1*, *CCNB1*, *CCND1* and (C) *p53* and *p21* mRNA expression levels ($n=4$). Data represent mean fold change compared to $0 \mu\text{M R} \pm \text{s.e.m.}$ * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to $0 \mu\text{M}$ of R, dotted line, fold change = 1.0). DC: decidualization cocktail; R: resveratrol.

LCB3II and p62 between 25 μM of R supplementation compared to $0 \mu\text{M}$ (Fig. 7).

These results indicate that the enhanced decidualization resulted from R supplementation is not accompanied by changes in the autophagic flux.

Discussion

Our present data demonstrate that R supplementation during *in vitro* decidualization of human ESC modifies – in a dose-dependent manner – the expression of several genes related to decidualization. In particular, we observed an increased expression of *PRL* and *IGFBP1*, indicating that R supplementation reinforces decidualization. Furthermore, we detected a decreased expression of key cell cycle regulators and a concomitant reinforcement of the inhibition of cell proliferation stimulated by the DC. In general, we found that R supplementation boosted the changes in gene expression stimulated in response to the DC, suggesting that R could be enhancing decidualization by reinforcing DC action in ESC.

Resveratrol and the expression of decidualization-related genes

Regarding the expression of secreted factors, R supplementation led to a dose-dependent increased

expression of *PRL* and *IGFBP1*. It has been previously described that the combined addition of *PRL* and *IGFBP1* increases cytotrophoblast invasion (Garrido-Gomez *et al.* 2017), indicating that the increased expression of these two secreted factors by decidual cells could contribute on its own to an improved embryo implantation. On the other hand, we found that R supplementation led to a decreased *IL-8* expression counter-acting the increased expression stimulated by the DC. Reduced expression of *IL-8* in different cell populations within the decidua has been associated with sporadic miscarriage in relation to normal cases (Pitman *et al.* 2013). It is important to notice that CD56+ uterine Natural Killer cells, an important endometrial source of growth factors and cytokines, macrophages and CD8+ T lymphocytes are also important decidual sources of *IL-8* (De Oliveira *et al.* 2010, Pitman *et al.* 2013). Therefore, the decreased expression of *IL-8* in ESC generated by R supplementation does not necessarily mean that trophoblast invasion will be impaired *in vivo*. Moreover, it has been described that a transient increase of *IL-8* expression during early decidualization could explain why more decidualized cells – upon supplementation with R – express less *IL-8* than less decidualized cells supplemented with vehicle (Brighton *et al.* 2017). Finally, the proangiogenic factor *VEGFA* was not modified by R supplementation, suggesting that R may not affect decidual vascularization.

Figure 6 Resveratrol supplementation during *in vitro* decidualization of HESC regulates mRNA expression of several genes associated with cell cycle progression. HESC were treated with DC for 3 days and then treated with DC plus different concentrations of R for 2 more days. Graphs show (A) *KI67*; (B) *CDC2*, *CDK2*, *CDK4*; (C) *CCNA1*, *CCNB1*, *CCND1* and (D) *p53* and *p21* mRNA expression levels ($n = 5$). Data represent mean fold change compared to 0 μM R $\pm$ S.E.M. ** $P < 0.01$; *** $P < 0.001$ (compared to 0 μM of R, dotted line, fold change = 1.0). DC: decidualization cocktail; R: resveratrol.

Regarding the expression of genes involved in cell signaling and gene expression regulation, R supplementation did not modify *FOXO1* mRNA expression, indicating that the important role of FOXO1 as a key transcription factor during decidualization was probably not affected (Kajihara *et al.* 2013). On the other hand, R supplementation reinforced the decreased expression of *NOTCH1* and *HOXA10* achieved by the DC in HESC; however, in t-HESC, R supplementation did not modify *NOTCH1* and *HOXA10* mRNA expression. The fact that the DC led to higher fold changes of *HOXA10* and *NOTCH1* in t-HESC than in HESC could explain why R supplementation did not further modify the expression of these genes in t-HESC (D vs ND for each cell type, Supplementary Fig. 2).

In this work, the increased expression of *PRL* and *IGFBP1* clearly indicates an increased level of differentiation of ESC and, furthermore, R supplementation seems to enhance the action of the DC during decidualization. Nevertheless, further experiments are needed to confirm in which degree the signaling pathways of the DC and R overlap and to

Figure 7 Effect of R supplementation over autophagic flux during t-HESC *in vitro* decidualization. t-HESC were treated with DC for 3 days and then treated with DC plus 25 μM R or vehicle for 2 more days (day 5). CQ (25 μM) was added 8 h before harvesting the cells. Figure shows representative immunoblots for protein levels of LC3B, p62 and GAPDH (internal control) ($n = 4$). Data represent mean relative protein expression to GAPDH $\pm$ S.E.M. DC: decidualization cocktail; R: resveratrol; CQ: chloroquine.

define the DC-independent signaling pathways that are activated by R during decidualization.

Resveratrol and cell cycle regulation

Another important finding of our study is the fact that proliferation was slightly diminished by R supplementation, reinforcing the reduction in cell proliferation stimulated by the DC. This effect of R on cell proliferation was more prominent in t-HESC than in HESC. It is expected that primary cells like HESC, with a limited lifespan, will be more sensitive to drugs affecting cell proliferation than immortalized cells like t-HESC. Particularly, in this work we saw that the fold change of *KI67* in D vs ND cells was much lower in HESC than in t-HESC. Therefore, it is not surprising that the effect of R over cell proliferation was more obvious in t-HESC, that maintain their proliferative capacity after the addition of the DC, than in HESC, that nearly completely lost *KI67* expression during decidualization.

Going into detail of R regulation of cell proliferation and cell cycle progression, we found that the expression of positive cell cycle regulators, like *CCNB1*, *CCND1* and different CDKs, was decreased during decidualization and further decreased in response to R supplementation. First, these results agree with previous studies describing a decreased expression of *CCND1*, *CDK2* and *CDC2* during ESC differentiation (Wang *et al.* 2018). And secondly, the fact that R was able to decrease even further the expression of these genes suggests a clear reinforcement by R of the DC regulation of cell cycle progression. Finally, we observed an increased expression of *CCNA1* in t-HESC and a decreased expression in HESC. *CCNA1* has not been

previously described in relation to decidualization, and its involvement in decidualization is still unclear after this study. Further experiments are needed to define if CCNA1 has a role during decidualization.

Regarding the expression of negative regulators of cell cycle progression, the expression of *p21* was slightly decreased by the DC in t-HESC and was highly increased in HESC. This difference could also be explained by the different proliferative capacity of both cell types, suggesting again that HESC are more sensitive to the anti-proliferative effect of the DC than t-HESC.

Overall, we suggest that the resulted balance of positive and negative cell cycle regulators that was turned in favor of the inhibition of cell cycle progression by the DC was further reinforced by R supplementation (Supplementary Fig. 4) (Logan *et al.* 2012, Casimiro *et al.* 2014, Wang *et al.* 2018). We would like to highlight the fact that R affects ESC proliferation is an important effect to consider, since a defect in cell proliferation could affect decidual extension. However, as this effect was less prominent in HESC than in t-HESC, we would expect that low doses of R supplemented *in vivo* will drive the changes in cell cycle regulators needed for an improved decidualization without affecting decidual extension. Nevertheless, an optimal time and dose for R *in vivo* supplementation is still to be defined to guaranty its safe use in the preparation of the uterus for a successful pregnancy, contributing to a proper decidualization without affecting the pre-decidual proliferation.

Resveratrol and endometrial health

It has recently been published that 100 μM of R inhibits *in vitro* decidualization (Ochiai *et al.* 2019). There are several differences between our experimental settings and the one described by Ochiai and colleagues. First, we used a lower range of R concentrations. Our highest concentration (50 μM) is half the concentration with which they found a significant reduction in the expression of decidualization markers. Furthermore, if we pay attention to the 50 μM dose (Figs 1 and 3A, B), we see that the expression of *PRL* and *IFGBP1* starts to decrease compared to lower doses. Therefore, it is possible that much higher R concentrations would lead to a deleterious effect on decidualization. Second, we used a lower concentration of cAMP. cAMP helps to catalyze faster the decidualization reaction (Brar *et al.* 1997) and we believed, and confirmed with this work, that lower doses of cAMP are more effective to study the action of combined drugs as, in this study, R supplementation during decidualization. Finally, we administered R to pre-decidualized cells (day 3 of DC treatment) instead to ND cells.

Taken together the results presented by Ochiai and colleagues and our present findings, R supplementation seems to have a biphasic effect on decidualization, a positive outcome for cell differentiation at lower doses

and a deleterious effect at higher doses. This biphasic effect of R has been previously described in adipocytes. In these cells, high doses of R (>20 μM) stimulate apoptosis and inhibit differentiation, while lower doses of R (1–10 μM) enhance *in vitro* adipocyte differentiation (Hu *et al.* 2015). It is worth to notice that for R action during adipocyte differentiation, the authors also utilized a weaker differentiation cocktail, as we described here for R supplementation during decidualization.

Interestingly, it has been also described that high doses of R are difficult to achieve physiologically (Boocock *et al.* 2007, Brown *et al.* 2010). Therefore, for the bench to bedside transition of R supplementation, it is important to define a physiologically achievable dose with a desirable effect for ESC function. In this same line of evidence are our present findings, as we were able to show that even the lower dose of R used (6.25 μM) was effective to modified gene expression in ESC during decidualization.

Regarding the signaling pathways described by Ochiai and colleagues, they found that mRNA expression of *BTG2*, an anti-proliferative gene, was increased during HESC decidualization and this increase was abrogated by 100 μM of R, indicating a positive regulation of cell proliferation upon R addition (Ochiai *et al.* 2019). This discrepancy with our results is expected as we are showing an opposite effect of R supplementation during decidualization. Furthermore, this result also supports the importance of focusing on proliferation and on the expression of cell cycle regulators while studying the effect of different drugs during decidualization. Besides, Ochiai and colleagues described that R-inhibitory action is mediated by SIRT1-dependent and SIRT1-independent mechanisms (Ochiai *et al.* 2019). Finally, they suggest that the accelerated down-regulation of the CRABP2-RAR pathway by R additionally mediates R-inhibitory action during decidualization. However, further experiments are still needed to define the precise signaling pathways activated by R in its biphasic effect during ESC decidualization.

In conclusion, our present work suggests that R supplementation could be beneficial for the correct decidualization of ESC. Further experiments are needed to define the signaling pathways activated by R and, in particular, to define the optimal dose, time of administration and length of the treatment for achieving a beneficial effect during *in vivo* decidualization. We are positive that the findings of this work will lead to a better understanding of endometrial function and will facilitate the identification and selection of new therapeutic options for fertility-related endometrial pathologies.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0425>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A C M C conceived the idea, designed and performed the experiments, analyzed the data and wrote the manuscript. A C M C, T S and A G provided funding. L L consented the patients. T S and A G contributed to the final version of the manuscript. A G supervised work.

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