Seminal Plasma Enhances and Accelerates Progesterone-Induced Decidualization of Human Endometrial Stromal Cells

U. Doyle[1], N. Sampson[1], C. Zenzmaier[1], P.Schwärzler[2], P. Berger[1]

[1]Institute of Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria.

[2]Obstetrics and Gynecology, Academic Teaching Hospital Feldkirch, Feldkirch, Austria.

Running Title: Seminal Plasma Potentiates Decidualization

Una Doyle, M.Sc.
Natalie Sampson, Ph.D.
Christoph Zenzmaier, Ph.D.
Peter Schwärzler, MD.
Peter Berger, Ph.D.

Corresponding Author:

Peter Berger Ph.D.
Institute of Biomedical Aging Research
Austrian Academy of Sciences
Rennweg 10
A-6020 Innsbruck
Austria
Phone: +43 512583919-24
Fax: +43 512 583919 8

Email: peter.berger@oeaw.ac.at

Disclosure Summary: The authors have nothing to disclose.

Supported By: National Research Network (NRN) Grant from the Austrian Science Fund (FWF) S9307-B05.

1 **ABSTRACT**

2

3 In preparation for embryo implantation endometrial stromal cells (ESC) undergo differentiation,
4 termed decidualization. Enhancing endometrial decidualization may overcome reduced
5 endometrial receptivity, a major limiting factor in natural and assisted reproduction. To determine
6 whether seminal plasma (SP) influences decidualization, primary human ESC were treated with
7 progesterone (P_4, 50 ng/ml) in the presence or absence of dialysed SP (0.5%) for 24 hours or for
8 up to 27 days to investigate immediate early effects or the effects of prolonged exposure,
9 respectively. Combined SP & P_4 treatment induced ESC morphological differentiation. Relative to
10 control, P_4 alone, and SP alone, combined treatment with SP & P_4 for 27 days significantly up-
11 regulated mRNA levels of the decidua-specific markers prolactin (PRL) and insulin-like growth
12 factor binding protein 1 (IGFBP1). Consistently, PRL protein secretion was significantly increased
13 over the course of 27 days combined SP & P_4 treatment relative to control, P_4 alone and SP alone.
14 Likewise, IGFBP1 secretion was significantly greater relative to control and P_4 alone over the
15 course of 27 days. Thus, SP enhances and accelerates P_4-mediated decidualization of human
16 ESC and may enhance endometrial receptivity.

17 **INTRODUCTION**

18

19 Although recent advances in the field of assisted reproduction and *in vitro* fertilization (IVF) have
20 significantly improved pregnancy rates, the maximum achievable pregnancy rate by assisted
21 reproduction is on average only 50% per cycle and cannot be increased further via alteration of
22 embryo culture or embryo transfer conditions (Gardner *et al.* 2000; Vlaisavljevic *et al.* 2001;
23 Strowitzki *et al.* 2006). Reduced endometrial receptivity is found in an increasing number of
24 unexplained infertilities. In preparation for implantation, the endometrium undergoes
25 decidualization, a process whereby the endometrium differentiates into a morphologically and
26 functionally distinct tissue of pregnancy, termed the decidua. The decidua is characterized by a
27 dense cellular matrix of polygonal cells, which arise via differentiation of fibroblast-like endometrial
28 stromal cells (ESC). In the absence of decidualization pregnancy cannot be maintained (Lim *et al.*
29 1997; Stewart and Cullinan 1997). Thus, an inadequately decidualized endometrium and thereby,
30 reduced receptivity is a major fertility limiting factor (Lessey *et al.* 1995; Strowitzki *et al.* 2006).

31

32 The success of decidualization is dependent upon two main criteria. Firstly, decidualization must
33 be precisely timed to the receptive phase of the reproductive cycle, the window of implantation.
34 Secondly, the ESC must be sufficiently decidualized to accommodate the implanting blastocyst.
35 Decidualization *in utero* occurs under the influence of the post-ovulatory rise in sex steroid
36 hormone progesterone (P_4). Decidualization results in the co-ordinated expression of decidua-
37 specific proteins, including prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1)
38 (Aplin *et al.* 2008). PRL and IGFBP1 appear sequentially as decidualization proceeds (PRL
39 precedes IGFBP1) and as such are employed as biochemical markers of decidualization (Aplin *et*
40 *al.* 2008).

41

42 Emerging evidence indicates that seminal plasma (SP) transferred to the endometrium at mating
43 elicits complex paracrine events in the human, rodents and the pig, including improved pregnancy
44 and implantation rates (Tremellen *et al.* 2000; Robertson 2005; Robertson 2007). However, the
45 effects of SP on decidualization have not yet been investigated. We hypothesized that SP may
46 alter the receptivity of the human endometrium by enhancing and/or accelerating decidualization.

MATERIALS & METHODS

Patients and cell collection

Human endometrial cells were obtained from pre-menopausal patients (n=10), without pre-cancerous conditions or malignant disease taking no hormonal medication at the time of surgery and undergoing hysterectomy for the treatment of benign disease such as bleeding disorders, uterine leiomyoma or uterine prolapse. The use of waste endometrial cells for scientific purposes was cleared by the human Ethics Committee of the State of Vorarlberg.

Semen samples for the purpose of infertility assessment/ treatment were obtained after 3-5 days of ejaculatory abstinence at the outpatient Andrology Unit, Hospital Hietzing, Vienna. Ejaculates from 88 individuals were processed according to World Health Organization recommendations (World Health Organization 1999) and residual SP stored at ≤ -70°C. After thawing SP were pooled, centrifuged (1000 x g) for 30 minutes at 4°C and dialyzed against PBS, using a 3500 molecular weight cut off dialysis membrane (Pierce), to remove low molecular weight components. Following dialysis SP pool was aliquoted and stored at -70°C until use.

Formal written informed consent was obtained from each patient.

Endometrial stromal cell culture

Human ESC were isolated using a modification of the method described by Moy et al. (Moy *et al.* 1996). Briefly, endometrium was minced and digested with 0.25% Collagenase (Sigma-Aldrich) in phenol red-free MEMα media (Gibco), supplemented with 4mg/ml D-glucose (Sigma-Aldrich) and 1% Penicillin/Streptomycin (PAA Laboratories) at 37°C for approximately 2 hours. Dissociated cells were incubated in the above media with 10 % charcoal-treated FBS (ctFBS) (HyClone) at 37°C, 5% CO_2 and ESC isolated by adherent selection after approximately 45 minutes. ESC were detached by trypsinization and plated at a density of 50-200,000 cells/well in 6-well plates (Falcon) in phenol red-free RPMI-1640 media (Invitrogen), containing 5% ctFBS, insulin (8 µg/ml), 2 mM L-glutamine (PAA Laboratories) and 1% Penicillin/Streptomycin. Once confluent, ESC were starved for 3 days to synchronize the cells akin to the first three days of the human menstrual cycle in the aforementioned RPMI-1640 containing 1% ctFBS (RPMI-1% ctFBS) (1 ml/well) prior to stimulation.

To investigate the effect of SP on human endometrial decidualization, ESC maintained in RPMI-1% ctFBS were treated for 24 hours to investigate immediate early effects or for up to 27 days at three-day intervals to investigate the effects of prolonged exposure with 50 ng/ml P_4 (Sigma-Aldrich) and/or 0.5% SP, or an equivalent volume of PBS (control). Decidualization was evaluated morphologically and by quantification of the decidua-specific markers PRL and IGFBP1 at the mRNA level by qPCR and protein level via sandwich immunoassay of the cell culture supernatants.

The media was exchanged completely at 3 day intervals over the course of 27 days culture. Supernatants were stored at -20°C until assayed for PRL and IGFBP1.

Replicate experiments (n > 5) were performed independently using ESC derived from different donors.

RNA extraction and real-time quantitative RT-PCR (qPCR)

RNA was isolated 24 hours and 27 days post-treatment using PeqGold Trifast™ (PeqLab) according to the manufacturer's instructions. cDNA was synthesized using Promega ImProm-II™ Reverse Transcriptase System from 2 µg total RNA. Thereafter, qPCR was performed to quantify mRNAs encoding the proteins hydroxymethylbilane synthase (HMBS), PRL, and IGFBP1 by Light-Cycler (Roche). Primer sequences were as follows: HMBS sense 5'-CCAGGACATCTTGGATCTGG-3', HMBS antisense 5'-ATGGTAGCCTGCATGGTCTC-3', PRL sense 5'-GAGATTGAGGAGCAAACCAA-3', PRL antisense 5'-GTAGGCAGTGGAGCAGGTTA-3', IGFBP1 sense 5'-GAGAGTTTAGCCAAGGCACA-3', IGFBP1 antisense 5'-GATCCTCTTCCCATTCCAAG-3'. The comparative threshold cycle (C_t) method was used to quantify IGFBP1 and PRL expression levels normalized to HMBS, a moderate copy number house-keeping gene not regulated under the experimental conditions employed (Livak and Schmittgen 2001). Whereby, the ΔC_t value was determined by subtracting the HMBS C_t value for each sample from the C_t value for each target gene.

Quantification of secreted PRL and IGFBP1

Cell culture supernatants were collected every third day for up to 27 days. Secreted PRL was measured in the supernatants using a two-site immunofluorometric assay as described previously (Untergasser *et al.* 2001). Assay sensitivity was 0.32 ng/ml, and the intra- and interassay co-efficients of variation were 3.2% and 3.6%, respectively.

Secreted IGFBP1 was measured in cell culture supernatants using a commercially available ELISA development kit (R&D Systems), as per manufacturers' protocol. Assay sensitivity was 0.06 ng/ml and the intra- and inter-assay co-efficients of variation were 6.3% and 8.3%, respectively.

Numerical data represent cumulative secreted PRL or IGFBP1 (ng/ml).

122 **Microscopy**

123

124 Morphological assessment of ESC was performed by microscopy of stimulated ESC after 27 days
125 of treatment using a Labovert FS (Leitz) microscope, PHACO 1a 160/- EF L 10/0.25 lens (Leica)
126 and GF 10x/ 20 eyepieces (Periplan) attached to a Coolpix E990 (Nikon) camera.

127

128 **Statistical analyses**

129

130 Data were analyzed using PASW Statistics 18 software. Cumulative PRL and IGFBP1 protein
131 secretion were analyzed using repeated measures analysis of variance (ANOVA) with log
132 transformation. qPCR data were analyzed using one way ANOVA. A Fishers LSD post-hoc test
133 was performed to verify significant differences between treatment groups. All graphical data
134 represent the mean ± SEM of at least five independent experiments using ESC derived from
135 different donors. A value of $P < 0.05$ was considered statistically significant.

136 **RESULTS**

137

138 **Seminal plasma & progesterone induces enhanced and accelerated decidualization**

139

140 Control treated ESC maintained a fibroblast-like appearance throughout the duration of the
141 experiment (Fig. 1A). In contrast, ESC stimulated with P_4 alone exhibited focal areas of polygonal
142 shaped cells, a phenotype characteristic of decidualization (Fig. 1B). P_4 treatment of ESC for 24
143 hours induced a trend toward PRL mRNA up-regulation (Table 1, Fig. 2), however compared to
144 control ESC this effect was not significant. In contrast, PRL mRNA levels were significantly
145 elevated after 27 days of P_4 treatment relative to control ($P < 0.05$) (Table 1, Fig. 2). Moreover, P_4
146 treated ESC secreted significantly ($P < 0.05$) more PRL relative to control cells (17 ng/ml *vs.* 4.4
147 ng/ml PRL) after 27 days (Fig. 3A). However, no significant changes in IGFBP1 mRNA or secreted
148 protein levels were observed following P_4 alone treatment over the course of 27 days relative to
149 control ESC (Table 1 and Fig. 3B).

150

151 ESC treated with SP alone were morphologically indistinguishable from control treated cells (Fig.
152 1C). Furthermore, there was no significant effect of SP alone on PRL or IGFBP1 mRNA levels, or
153 PRL secretion after 24 hours or 27 days compared to control (Table 1, Fig. 2 and Fig. 3A).
154 However, SP alone induced a slight, but overall non-significant ($P = 0.070$) increase in secreted
155 IGFBP1 protein levels relative to control ESC (5.5 ng/ml *vs.* 0.5 ng/ml 27 day IGFBP1 secretion)
156 and a significant ($P < 0.01$) increase compared to P_4 (0.6 ng/ml IGFBP1) (Fig. 3B).

157

158 In contrast, SP & P_4 combined treatment induced extensive phenotypic ESC differentiation (Fig.
159 1D). Despite a tendency toward PRL and IGFBP1 mRNA up-regulation after 24 hours, levels were
160 not significantly elevated relative to PBS control treated ESC (Table 1, Fig. 2). Similarly, IGFBP1
161 mRNA levels were not significantly different after 24 hours. However, after 27 days SP & P_4
162 combined treatment PRL and IGFBP1 mRNA levels were up regulated compared to 24 hours
163 combined stimulation ($P < 0.01$ and $P < 0.001$, respectively). Moreover, the induction of PRL
164 mRNA after 27 days combined treatment was significantly higher than PBS control treated ESC (P
165 < 0.001), P_4 ($P < 0.05$), and SP alone ($P < 0.01$). Similarly, IGFBP1 mRNA levels were significantly
166 induced after 27 days combined treatment compared to control ($P < 0.001$), P_4 alone ($P < 0.01$), or
167 SP alone ($P < 0.05$). Consistent with up-regulation of PRL mRNA levels, PRL secretion was
168 significantly increased upon combined SP & P_4 treatment (27 day secretion: 427 ng/ml) compared
169 to PBS control ESC (4.4 ng/ml; $P < 0.001$) and significantly greater than the induction with P_4 alone
170 (17 ng/ml; $P < 0.01$), or SP alone (6.5 ng/ml; $P < 0.001$) (Fig. 3A). Furthermore, PRL secretion
171 upon SP & P_4 combined treatment induced a significant increase in PRL secretion earlier (day 12)
172 than compared to P_4 (day 18). Similarly, IGFBP1 secretion increased upon combined SP & P_4
173 treatment relative to PBS control treated ESC (10 ng/ml *vs.* 0.5 ng/ml; $P < 0.01$), P_4 alone (0.6
174 ng/ml; $P < 0.001$) and SP alone stimulated ESC (5.5 ng/ml; $P = 0.079$) (Fig. 3B). Compared to P_4

175 alone IGFBP1 secretion was significant from day 3 onwards upon SP & P_4 combined treatment
176 (day 3: 0.01 *vs.* 0.18 ng/ml; $P < 0.01$).

177 **DISCUSSION**

178

179 This study aimed to investigate the potential role of SP in human ESC decidualization. We
180 demonstrate that ESC treated with SP & P_4 in combination undergo phenotypic differentiation,
181 exhibiting a polygonal morphology characteristic of decidualization. Moreover, SP synergistically
182 enhanced and accelerated P_4-mediated decidualization as determined by induction of the decidua-
183 specific markers IGFBP1 and PRL at the mRNA and secreted protein level. To our knowledge, this
184 is the first report describing a functional role of SP in the process of human ESC decidualization.

185

186 It is established that SP deposited in rodents and pigs elicits complex molecular and cellular
187 changes that favor conception and pregnancy. This inductive action of SP is considered to
188 primarily involve immuno-modulatory mechanisms (Robertson *et al.* 1998; Robertson *et al.* 2002;
189 Robertson 2005; Robertson 2007). Although few studies have specifically investigated the
190 influence of SP on female fertility in humans, significantly improved pregnancy rates have been
191 reported in women undergoing IVF that are exposed to SP via sexual intercourse (Tremellen *et al.*
192 2000). Moreover, the use of SP pessaries by women experiencing recurrent miscarriage of
193 unknown cause improves implantation rates (Coulam and Stern 1995). Collectively, these findings
194 indicate that SP has beneficial effects on rodent, porcine and human reproductive success;
195 however the precise underlying mechanism(s) remain to be resolved. The data presented herein
196 suggest that further to its potential immuno-modulatory function, SP may act as a specific
197 decidualizing agent in the human endometrium.

198

199 Several constituents of SP are reported to regulate PRL production, including human chorionic
200 gonadotropin (hCG) and α-subunit thereof, transforming growth factor beta (TGFβ), relaxin and
201 prostaglandin E_2 (PGE_2) (Tabanelli *et al.* 1992; Frank *et al.* 1994; Lane *et al.* 1994; Moy *et al.* 1996;
202 Brar *et al.* 1997; Bartsch and Ivell 2004; Chang *et al.* 2008). However, the magnitude of PRL
203 mRNA and protein secretion induction by hCG, hCGα or TGFβ, neither alone nor in combination
204 with P_4 was commensurate with PRL induction upon combined SP & P_4 treatment (own
205 unpublished observations). Moreover, the current study employed dialyzed SP to deplete low
206 molecular weight components such as PGE_2. Thus, the molecular component(s) of SP that
207 potentiate P_4-mediated endometrial decidualization remain to be identified.

208

209 We speculate that coital endometrial exposure to SP arises as a consequence of peristaltic
210 contractions that transport sperm and hence SP to the uterine cavity. The concentration of
211 secreted PRL after 27 days of combined SP & P_4 treatment (427 ng/ml) is comparable to PRL
212 levels detected in amniotic fluid (151 ng/ml) (Hernandez-Andrade *et al.* 2005). Amniotic fluid PRL is
213 a product of maternal decidualized endometrium (Coshen 1989). Thus, although potentially minute
214 amounts of SP reach the uterine cavity the low SP concentration (0.5%, which represents EC_{50} of
215 PRL secretion under combined SP & P_4 treatment, data not shown) employed herein induced a
216 PRL response of physiological magnitude.

217

218 Decidualization is characterized by the post-ovulatory rise in P_4, however phenotypic differentiation
219 is only first apparent during the mid-secretory phase of the reproductive cycle (approximately 10
220 days after the post-ovulatory rise in P_4) (Gellersen and Brosens 2003; Aplin *et al.* 2008). Thus, it is
221 unlikely that the decidua-specific markers PRL and IGFBP1 are under direct transcriptional control
222 of the P_4 receptor *in vivo* (Aplin *et al.* 2008). Supportively, we demonstrate herein that P_4 induces a
223 relatively weak morphological decidual response with up-regulation of PRL and IGFBP1 at the
224 mRNA and protein level. A similar finding was also observed *in vivo* (Brar *et al.* 1997). Collectively,
225 these data suggest that P_4 alone would induce an asynchronous and inadequately primed decidua
226 in the window of implantation. Thus, P_4 appears to act as a permissive factor rather than an active
227 decidualizing agent. In contrast, secreted PRL protein levels upon 12 days of combined SP & P_4
228 treatment were comparable to those after 27 days treatment with P_4 alone. Moreover, ESC treated
229 with SP & P_4 in combination for 27 days secreted significantly higher concentrations of IGFBP1 and
230 PRL than ESC treated with either agent alone. Thus, under the combined influence of SP & P_4
231 ESC decidualization is accelerated and the magnitude of decidua-specific IGFBP1 and PRL
232 induction greater.

233

234 Reduced endometrial receptivity is observed in an increasing number of unexplained infertilities
235 (Strowitzki *et al.* 2006). Consequently, an inadequately decidualized endometrium is considered a
236 major fertility-limiting factor (Strowitzki *et al.* 2006). Since embryo implantation for the
237 establishment of pregnancy typically occurs 6-12 days post-ovulation, a sufficient decidual
238 response during the "window of implantation" is critical (Wilcox *et al.* 1999; Strowitzki *et al.* 2006).
239 In assisted conception decidualization is routinely induced via intra-vaginal P_4 pessaries. P_4
240 absorbed locally is expected to support the luteal phase and thus intensify endometrial
241 decidualization. However, a randomized controlled study of pregnancy rates following IVF
242 indicated no significant difference in pregnancy rates following the administration of P_4 pessaries
243 (Polson *et al.* 1992). Consequently, there is an urgent need for improved decidualizing agents in
244 assisted reproduction. In this respect, the capacity of SP to potentiate P_4-mediated human ESC
245 decidualization is potentially of significant clinical relevance for the field of reproductive medicine.

246

247 In summary, our model provides a useful conceptual framework linking the events of
248 decidualization with exposure to SP and uterine receptivity. A better understanding of the
249 processes underlying decidualization and the molecular basis by which SP potentiates P_4-
250 mediated decidualization may facilitate the development of therapeutic strategies to improve
251 pregnancy outcome. In this respect, the active component(s) of SP could be employed in
252 combination with P_4 pessaries as a decidualizing agent in a clinical setting to complement natural
253 and assisted reproductive therapies.

254 **ACKNOWLEDGEMENTS**

255

256 We gratefully thank Dr. Eugen Plas (Department of Urology, Ludwig-Boltzmann Institute for
257 Urology and Andrology, Hospital Hietzing, Vienna, Austria) for providing post-semen analysis
258 seminal plasma and Regine Gerth for initial technical assistance.

259 **REFERENCES**

260

261 Aplin, J. D., Fazleabas, A. T., Glasser, S. R., Giudice, L. C. (Eds). (2008). 'The endometrium:
262 Molecular, cellular and clinical perspectives (2nd edn).' (Informa, UK: London)

263

264 Bartsch, O., Ivell, R. (2004). Relaxin and phosphodiesterases collaborate during decidualization.
265 *Ann. N. Y. Acad. Sci.* **1030**, 479-492.

266

267 Brar, A. K., Frank, G. R., Kessler, C. A., Cedars, M. I., Handwerger, S. (1997). Progesterone-
268 dependent decidualization of the human endometrium is mediated by camp. *Endocrine.* **6** (3), 301-
269 307.

270

271 Chang, H. J., Lee, J. H., Hwang, K. J., Kim, M. R., Chang, K. H., Park, D. W., Min, C. K. (2008).
272 Transforming growth factor (tgf)-beta1-induced human endometrial stromal cell decidualization
273 through extracellular signal-regulated kinase and smad activation in vitro: Peroxisome proliferator-
274 activated receptor gamma acts as a negative regulator of tgf-beta1. *Fertil. Steril.* **90** (4 Suppl),
275 1357-1365.

276

277 Coshen, A. (1989). Fetal control of maternal prolactin production and bioactivity in utero. *Am. J.*
278 *Obstet. Gynecol.* **160** (2), 322-327.

279

280 Coulam, C. B., Stern, J. J. (1995). Effect of seminal plasma on implantation rates. *Early*
281 *Pregnancy.* **1** (1), 33-36.

282

283 Frank, G. R., Brar, A., K, Cedars, M., I , Handwerger, S. (1994). Prostaglandin e2 enhances
284 human endometrial stromal cell differentiation. *Endocrinology.* **134**, 258-263.

285

286 Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., Schoolcraft, W. B. (2000). Blastocyst score
287 affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil. Steril.* **73**
288 (6), 1155-1158.

289

290 Gellersen, B., Brosens, J. (2003). Cyclic amp and progesterone receptor cross-talk in human
291 endometrium: A decidualizing affair. *J. Endocrinol.* **178** (3), 357-372.

292

293 Hernandez-Andrade, E., Villanueva-Diaz, C., Ahued-Ahued, J. (2005). Growth hormone and
294 prolactin in maternal plasma and amniotic fluid during normal gestation. *Rev Invest Clin.* **57** (5),
295 671-675.

296

297 Lane, B., Oxberry, W., Mazella, J., Tseng, L. (1994). Decidualization of human endometrial stromal
298 cells in vitro: Effects of progestin and relaxin on the ultrastructure and production of decidual
299 secretory proteins. *Hum Reprod.* **9** (2), 259-266.
300
301 Lessey, B. A., Castelbaum, A. J., Sawin, S. W., Sun, J. (1995). Integrins as markers of uterine
302 receptivity in women with primary unexplained infertility. *Fertil. Steril.* **63** (3), 535-542.
303
304 Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., Dey, S. K.
305 (1997). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell.* **91** (2), 197-
306 208.
307
308 Moy, E., Kimzey, L., Nelson, L., Blithe, D. (1996). Glycoprotein hormone alpha-subunit functions
309 synergistically with progesterone to stimulate differentiation of cultured human endometrial stromal
310 cells to decidualized cells: A novel role for free alpha-subunit in reproduction. *Endocrinology.* **137**
311 (4), 1332-1339.
312
313 Polson, D. W., Rogers, P. A., Krapez, J. A., Leeton, J. F. (1992). Vaginal progesterone as luteal
314 phase support in an ivf/gift programme. *Eur J Obstet Gynecol Reprod Biol.* **46** (1), 35-38.
315
316 Robertson, S. A. (2005). Seminal plasma and male factor signalling in the female reproductive
317 tract. *Cell Tissue Res.* **322** (1), 43-52.
318
319 Robertson, S. A. (2007). Seminal fluid signaling in the female reproductive tract: Lessons from
320 rodents and pigs. *J. Anim. Sci.* **85** (13 Suppl), E36-44.
321
322 Robertson, S. A., Allanson, M., Mau, V. J. (1998). Molecular regulation of uterine leukocyte
323 recruitment during early pregnancy in the mouse. Troph. *Res.* **11**, 101-120.
324
325 Robertson, S. A., Ingman, W. V., O'Leary, S., Sharkey, D. J., Tremellen, K. P. (2002).
326 Transforming growth factor beta--a mediator of immune deviation in seminal plasma. *J. Reprod.*
327 *Immunol.* **57** (1-2), 109-128.
328
329 Stewart, C. L., Cullinan, E. B. (1997). Preimplantation development of the mammalian embryo and
330 its regulation by growth factors. *Dev. Genet.* **21** (1), 91-101.
331
332 Strowitzki, T., Germeyer, A., Popovici, R., von Wolff, M. (2006). The human endometrium as a
333 fertility-determining factor. *Hum. Reprod. Update.* **12** (5), 617-630.
334
335 Tabanelli, S., Tang, B., Gurpide, E. (1992). In vitro decidualization of human endometrial stromal
336 cells. *J. Steroid Biochem. Mol. Biol.* **42** (3-4), 337-344.

337
338 Tremellen, K. P., Valbuena, D., Landeras, J., Ballesteros, A., Martinez, J., Mendoza, S., Norman,
339 R. J., Robertson, S. A., Simon, C. (2000). The effect of intercourse on pregnancy rates during
340 assisted human reproduction. *Hum Reprod.* **15** (12), 2653-2658.
341
342 Untergasser, G., Rumpold, H., Plas, E., Witkowski, M., Berger, P. (2001). Seminal plasma factors
343 induce in vitro prl secretion in smooth muscle cells of the human prostate. *J. Clin. Endocrinol.*
344 *Metab.* **86** (11), 5577-5584.
345
346 Vlaisavljevic, V., Kovacic, B., Reljic, M., Lovrec, V., Sajko, M. (2001). Is there any benefit from the
347 culture of a single oocyte to a blastocyst-stage embryo in unstimulated cycles? *Hum Reprod.* **16**
348 (2379-2383).
349
350 Wilcox, A. J., Baird, D. D., Weinberg, C. R. (1999). Time of implantation of the conceptus and loss
351 of pregnancy. *N. Engl. J. Med.* **340** (23), 1796-1799.
352
353 World Health Organization. (1999). 'Who laboratory manual for the examination of human semen
354 and sperm-cervical mucus interaction.' 4th edn. (Cambridge University Press: Cambridge)

FIGURE LEGENDS

Figure 1

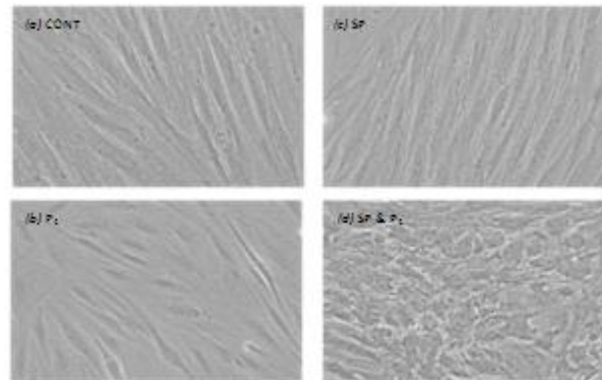

Figure 1. Morphology of human endometrial stromal cells upon progesterone and/or seminal plasma stimulation.

Primary human endometrial stromal cells (ESC) stimulated for 27 days with *(a)* PBS control, *(b)* progesterone (P_4), *(c)* seminal plasma (SP), or *(d)* SP & P_4 combined (SP & P_4). Images are representative of at least three independent experiments after 27 days of treatment (original magnification 100x).

Figure 2

Figure 2. PRL and IGFBP1 gene expression in response to stimulation of human endometrial stromal cells with progesterone and/or seminal plasma.

Agarose gel electrophoresis of PRL or IGFBP1 quantitative PCR products from EnSC incubated for the duration indicated with PBS control (CONT), progesterone (P_4), seminal plasma (SP) or a combination of both (SP & P_4) processed for quantitative PCR of PRL or IGFBP1 and PCR terminated in the logarithmic amplification phase. Amplification products of HMBS, a moderate copy number house-keeping gene, are shown as loading control. Images are representative of three independent experiments using EnSC derived from different donors.

Figure 3

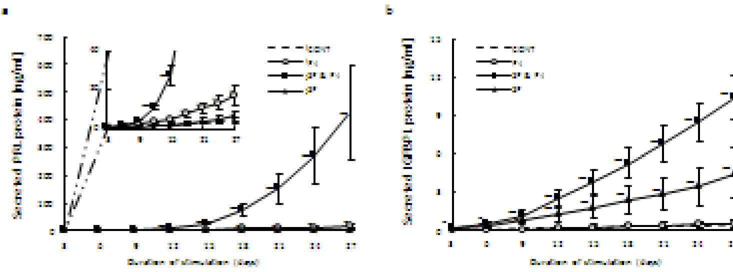

Figure 3. PRL and IGFBP1 secretion in response to stimulation of human endometrial stromal cells with progesterone and/or seminal plasma.

Secretion of *(a)* PRL, or *(b)* IGFBP1 by endometrial stromal cells (ESC) treated for the duration indicated with PBS control (CONT), progesterone (P_4), seminal plasma (SP), or a combination of both (SP & P_4). Values represent mean cumulative secreted PRL or IGFBP1 protein levels ± SEM (n = 5). *$P < 0.05$, **$P < 0.01$ indicates statistical significance *vs.* control.

Table I

Quantitative PCR of PRL and IGFBP1 in ESC incubated for the duration indicated with PBS control (CONT), progesterone (P_4), seminal plasma (SP) or a combination of both (SP & P_4). The ΔC_t values were determined by subtracting the target gene C_t value for each sample from the HMBS, a moderate copy number house-keeping gene, C_t value. Different letters indicate statistical significance where $P \leq 0.05$ ($n = 5$).

	Δ C_t PRL				**Δ C_t IGFBP1**			
	24 hrs		**27 days**		**24 hrs**		**27 days**	
Sample	**Mean ± SEM**	**Median**	**Mean ± SEM**	**Median**	**Mean ± SEM**	**Median**	**Mean ± SEM**	**Median**
CONT	-8.50 ± 1.26[a]	-8.14	-8.04 ± 1.10[a]	-7.21	-5.22 ± 0.57[a]	-5.17	-6.66 ± 0.73[a]	-5.89
P_4	-8.43 ± 0.94[a]	-8.07	-4.47 ± 0.84[b]	-5.54	-6.13 ± 0.47[a]	-6.16	-5.32 ± 0.61[a]	-5.68
SP & P_4	-7.11 ± 1.49[ab]	-7.03	0.16 ± 1.45[c]	-2.32	-4.40 ± 0.77[a]	-3.99	1.44 ± 1.78[b]	2.81
SP	-7.57 ± 1.68[ab]	-6.86	-6.57 ± 1.04[ab]	-7.35	-3.26 ± 1.05[a]	-3.17	-3.28 ± 1.30[a]	-3.11