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The effect of clomiphene citrate

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CHAPTER 4: DISCUSSION

4.1 IMPORTANCE OF OVARIAN HORMONES ON RECEPTIVITY TO IMPLANTATION IN THE UTERUS

It has been well established that, except for a brief period of time in the menstrual or estrus cycle of mammals, the uterus remains in a state whereby it is unable to allow fertilized ova to implant into its wall (Psychoyos, 1973; Finn and Porter, 1975). Currently, not much is known as to how this change in receptivity occurs, especially at the gene expression level. However, it is almost certainly under the control of the maternal ovarian hormones E_2 and P_4 (Yoshinaga and Adams, 1966a; Psychoyos, 1973; 1976; Psychoyos and Martel, 1985; Paulson *et al.,* 1990).

4.1.1 Histological Changes between the 5½ Day Pregnant and PPPE Treated Rat Uteri

In the current study, it is observed that in the H&E sections (Fig.3.9), the morphology of the 5½ day pregnant (Group G) and PPPE (Group H) treated rat uteri is very similar. Both uterine sections have a large diameter with a slightly convoluted lumen, which is lined by columnar epithelium. The luminal space is also small, with the apical surfaces of the luminal epithelium almost touching each another. All of these morphological features have been observed in previous studies and have been suggested to be due to the ovarian hormones acting on the stroma and/or luminal epithelium, in preparation for implantation and pregnancy (Clark *et al.,* 1974; Finn and Porter, 1975; Knobil and Neill, 1994; Hewitt and Korach, 2000).

Since the histology of the 5½ day pregnant rat uteri (G), which is known to be receptive to implantation, is similar to the PPPE treated rat uteri (H), it supports the theory that it is the sequence of maternal P_4 and E_2 that determines whether the uterus is receptive to implantation, rather than factors contributed by the blastocyst (Yoshinaga and Adams, 1966a; Psychoyos, 1973; Psychoyos and Martel, 1985; Paulson *et al.,* 1990).

The only histological differences observed between the PPPE treated (Fig.3.9H) and pregnant (Fig.3.9G) sections, are that the uterine lumen of the pregnant section is not as convoluted as in the PPPE treated section and that there is no stromal edema present in the PPPE treated rat uteri, whereas there is in the 5½ day pregnant rat uteri.

In the single dose treated H&E sections (Fig.3.3), it is observed that E_2 (Group E) can induce a more convoluted lumen in the rat uterus, than in the uteri of rats without hormone stimulation (Group A [untreated] and Group C [oil treated]). This has also been shown in previous studies and has been suggested to be a result of hypertrophy (increase in cell height) and hyperplasia (increase in cell proliferation) of the luminal epithelium, in response to E_2 (Poteat and Bo, 1977; Hosie and Murphy, 1992; 1995). It is therefore possible that the nidatory dose of E_2 used in the PPPE treated samples (Fig.3.9H) was too high in comparison to the natural nidatory surge of E_2 in the pregnant sample (Fig.3.9G), thus producing a more convoluted lumen in the PPPE treated rat uteri than in the pregnant rat uteri.
Although higher doses of E_2 have been used, as the nidatory E_2 in P_4 primed uteri, to induce implantation, generally lower doses are used such as 0.25μg and even 0.1 μg (Ljungkvist, 1972; Psychoyos, 1973; Hammer *et al.,* 1978; Ma *et al.*, 2003). Therefore a lower dose may have produced a closer resemblance to the pregnant sample (G). Higher doses of E_2 have also been shown to shorten the window of receptivity, in P_4 primed rat uteri, compared to lower doses of E_2, even though both are capable of inducing receptivity to implantation of donor embryos (Ma *et al.,* 2003). In future studies it is suggested that a lower dose of E_2 should be used to induce implantation in ovariectomized P_4 primed uteri.

The observation that there is stromal edema in the pregnant rat uteri (Fig.3.9G), whereas there is a lack of stromal edema in the PPPE treated rat uteri (Fig.3.9H), suggests that uterine stromal edema may be influenced by factors produced by the implanting blastocyst, in the pregnant rat uteri, as well as by E_2 and P_4. In support of this decidualization, in which stromal edema is a major characteristic, can be induced by a mechanical stimulus, in addition to priming the uterus with P_4 (Knobil

and Neill, 1994; Hewitt and Korach, 2000). Such a mechanical stimulus would be expected to be produced by an implanting blastocyst, which is not present in the PPPE (Group H) and CCPPPE (Group I) treated samples. Therefore, this would account for the absence of stromal edema in these uteri (Fig.3.9). It also suggests that stromal edema, and therefore decidualization, is not required for the initial attachment of the blastocyst to the uterus and for receptivity of the uterus to this attachment. However, it does not dispute the importance of decidualization in later stages of implantation and in maintenance of pregnancy (Knobil and Neill, 1994; Hewitt and Korach, 2000).

4.1.2 Similarity in Expression of ERα, Hsp90 and Hoxa10 between the 5½ Day Pregnant and PPPE Treated Rat Uteri

As with the H&E results, there appears to be no difference between the 5½ day pregnant (G) and PPPE treated (pseudopregnant) (H) rat uteri in the location, protein expression and/or mRNA expression of ERα (Figs.3.10; 3.7(I); 3.8(I); 3.12; and Table 3.2), Hsp90 (Figs.3.11; 3.7(II); and 3.8(II)) and Hoxa10 (Fig.3.15) at the time of implantation, as determined by fluorescence ICC, Western blotting and/or RT-PCR. This suggests that the level of expression of ERα, Hsp90 and Hoxa10, in the rat uterus at implantation, is directly related to the ovarian hormones present, and not due to the presence or absence of the blastocyst, as no blastocyst was present in the PPPE treated rat uteri (H). This result supports previous suggestions, by other investigators, that the success or failure of implantation is due to the particular sequence of ovarian hormones that condition the uterus for implantation, rather than factors contributed by the blastocyst (Yoshinaga and Adams, 1966a; Psychoyos, 1973; Psychoyos and Martel, 1985; Paulson *et al.,* 1990). The present study is also the first to show this at the gene expression level, whereas previous studies have shown this at the ultrastructural, histological and morphological levels (Ljungkvist, 1971a; 1971b; 1971c; 1972; Psychoyos, 1973).

4.2 ESTROGEN RECEPTOR ALPHA EXPRESSION IN THE RAT UTERUS

4.2.1 Effect of a Single Nidatory Dose of E_2 on ERα Expression in the Rat Uterus

Western blotting (Figs.3.1(I) and 3.2(I)), fluorescence ICC (Fig.3.4) and RT-PCR (Table 3.1 and Fig.3.6) showed that a single dose of E_2 (E) increases ERα protein and mRNA, in the ovariectomized rat uterus, 24 hours after its administration. These results correlate with previous studies, in which ERα levels increased 18, 24 and 48 hours after the administration of a single dose of E_2, in sheep and rat uteri (Robertson *et al.,* 1998; Wang *et al.,* 1999). However similar studies in uterine, breast and pituitary tissue, have indicated that E_2 decreases the expression of ER in these cells (Horigome *et al.,* 1988; Martin *et al.,* 1994; Alarid *et al.,* 1999; Nawaz *et al.,* 1999). Although these studies appear to contrast with the results obtained in the present study, it is observed that the level of ER expression was determined only a few (less than eight) hours after the administration of E_2.

It is therefore of interest that a few studies have shown a decrease in ER (or ERα) levels, in the rat uterus, four to six hours after a single dose of E_2, followed by an increase 12, 18 and/or 24 hours after treatment (Rosser *et al.,* 1993; Zhou *et al.,* 1993; Nephew *et al.,* 2000). All together these results suggest that a single dose of E_2 has a biphasic effect on the regulation of its receptor in the uterus.

Estradiol 17β has also been shown to result in an initial decrease and later increase in the expression of ERα in human endothelial cells (Ihionkhan *et al.,* 2002). This may suggest that the biphasic regulation of ERα levels is not only restricted to the uterus, but may be a common mechanism of regulating the effects of E_2 in a number of different tissues.

A single dose of E_2 has also been shown to have a biphasic effect on collagen structure and organization in the rat uterus (Pastore *et al.,* 1989). The results of that study showed that there was an initial loss of collagen bundles 4-6 hours after E_2 administration and then a reappearance of collagen is observed, with a different

structure and organization, 24 and 48 hours after the initial E_2 treatment (Pastore *et al.,* 1989). It is therefore possible that the initial depletion of collagen bundles, could result from the initial depletion in ERα levels and subsequent estrogen signaling, within the uterus. Similarly the reappearance of collagen, 24 hours after the original E_2 treatment, could result from the later increase in ERα levels and estrogen signaling within the cell. Therefore the biphasic regulation of ERα by E_2 may be required to remodel the structure and organization of the uterus, during the estrus cycle and during pregnancy.

4.2.1.1 Mechanism of E_2 induced biphasic regulation of ERα in the uterus

The mechanism in which the biphasic change in ERα levels occurs, is not completely elucidated. However, it is generally thought that the initial decrease in ERα levels, in response to E_2 treatment, occurs post transcriptionally, through degradation of the receptor by the ubiquitin proteasome pathway (Rosser *et al.,* 1993; Zhou *et al.,* 1993; Alarid *et al.,* 1999; Nawaz *et al.,* 1999; Lonard *et al.,* 2000; Ihionkhan *et al.,* 2002).

The increase in the mRNA level of ERα in the rat uterus, observed in this study (Table 3.1 and Fig.3.6) and in those by Rosser *et al.,* (1993) and Nephew *et al.,* (2000), suggests that new ERα protein is then synthesized 24 hours after E_2 administration, instead of being recycled, as previously suggested (Baudendistel *et al.,* 1978; Adashi *et al.,* 1980; Kassis and Gorski, 1981). As there appears to be no estrogen response element (ERE) in the ERα gene (Nephew *et al.,* 2000), this increase in ERα levels does not occur through direct binding of the ligand-bound ER to the promoter region of its own gene. It is therefore possible that E_2 first induces the transcription of another transcription factor(s), which in turn induces the transcription of ERα. This would account for the delay in increasing ERα levels in the cell following E_2 treatment, and in the mean time, the delay allows the ERαs already present in the cell, to be degraded by the ubiquitin proteasome pathway.

Since the ERα levels ultimately increase in the cell following E_2 treatment, the initial decrease may seem unnecessary. However, Lonard *et al.,* (2000) has suggested

that the degradation of ERα by the ubiquitin proteasome pathway may be required for ERα to serve as an activated transcription factor, by enabling the receptor to interact with coactivators. In addition, the ubiquitin proteasome pathway has been linked to other processes, such as cell cycle regulation, signal transduction and differentiation, all of which are known to be mediated by ERα (Hershko and Ciechanover, 1998; Nawaz *et al.,* 1999). Therefore E_2 first stimulates the degradation of the receptor a few hours after its administration, by the ubiquitin proteasome pathway, in order to stimulate the transcriptional activity of the ERα. Following this, new ERα protein synthesis is stimulated to overcome the initial decrease in ERα levels and then to ensure a greater sensitivity of the uterus, to any subsequent E_2.

4.2.2 Estrogen Receptor Alpha Expression During Uterine Receptivity to Implantation in the Rat Uterus

4.2.2.1 Effect of P_4 on ERα expression

In terms of gene expression at implantation, although the present study may suggest that a nidatory dose of E_2 produces a biphasic effect, on ERα expression in the rat uterus, when administered alone, the effects of P_4 were not taken into account, in the single dose treatments (E) and in previous studies observing an E_2 induced biphasic regulation of ERα (or ER) expression (Rosser *et al.,* 1993; Zhou *et al.,* 1993; Nephew *et al.,* 2000). Progesterone has also been shown to be crucial in inducing a receptive uterus for implantation (Psychoyos, 1973; 1976), and has been shown to influence the effects of E_2, when both hormones are administered together (Graham and Clarke, 1997; Hewitt and Korach, 2000).

In the current study, the Western blot (Figs.3.7(I) and 3.8(I)), fluorescence ICC (Fig.3.10) and RT-PCR (Table 3.2 and Fig.3.12) results all indicate that there is no significant difference between the expression of ERα between the non-receptive (SOOO treated) (Group F) and the receptive (PPPE treated) (Group H) rat uteri. This suggests that the increase in ERα expression, following a single dose of E_2 (E), may be counteracted, or inhibited, by the P_4 used to prime the uterus before E_2

administration in the PPPE treated (H) samples. Therefore this would result in a similar expression of ERα, following PPPE treatment, to that before treatment, as observed in the multiple dose vehicle treated control (SOOO treated) rat uteri (F).

In support of this theory, it has been shown that ER levels are generally low, following implantation in pregnant baboon uteri, as a result of increasing P_4 levels (Hild-Petito *et al.,* 1992). Progesterone has also been shown to decrease ER immunostaining in the endometrium of ovariectomized primates (Hild-Petito *et al.,* 1992). In addition, P_4 has been shown to dampen the effects of E_2, by reducing the increase in uterine weight and lactoferrin expression, induced by E_2, when both hormones are administered together in mice (Hewitt and Korach, 2000). Therefore P_4 appears to influence the expression of genes downstream of the ER, and hence supports the possibility of cross-talk, between the signaling pathways of the ER and progesterone receptor (PR) (Graham and Clarke, 1997; Hewitt and Korach, 2000).

4.2.2.2 Estrogen Receptor Alpha levels at implantation in the rat uterus

The similarity in ERα expression between the non-receptive (SOOO treated) (F) and the receptive (PPPE treated) (H) rat uteri (Figs.3.7(I); 3.8(I); 3.10; Table 3.2 and Fig.3.12) may also suggest that ERα is not a marker of receptivity, or rather that a change in ERα expression is not required to induce receptivity, in the rat uterus, for implantation.

In support of a change in ERα not being required to confer receptivity on the uterus to implantation, previous studies have shown that there is no difference in ER immunostaining between the implantation and non-implantation sites, of primate endometria, during early pregnancy (Hild-Petito *et al.,* 1992; Puri *et al.,* 2000). Estrogen Receptor expression has also been shown to remain unaltered in primate endometrium when treated with various concentrations of an antiprogestin, onapristine, that causes the endometrium to become non-receptive, without affecting ovulation, corpus luteum function and menstrual cycle length (Puri *et al.,* 2000).

Studies by Tan *et al.,* (1999) have also shown that, in general, the levels of ERα mRNA and protein did not vary much, in the whole uterus, between days 1-8 of pregnancy in the mouse. However an increase in ERα immunostaining was observed in the uterine glandular and luminal epithelium and in the subluminal stroma at the mesometrial pole on day 5 of pregnancy, following implantation, in the mouse, whereas at the antimesometrial pole ERα immunostaining was less intense in the decidualizing stroma, at the site of implantation (Tan *et al.,* 1999).

Such a localized difference in expression, may account for why the results in the present study indicate that there is no change in expression of ERα between the SOOO (F) and PPPE (H) treated groups (Figs.3.7(I); 3.8(I); 3.10; Table 3.2; and Fig.3.12). This is because the whole uterus was used and therefore did not account for changes within the uterine compartments. It is suggested that, in future studies, the luminal epithelium, glandular epithelium, stroma and myometrium, should be separated and the expression of ERα quantified between these three uterine compartments.

4.2.2.3 Biphasic regulation of ERα and implantation

It is important to note that none of the previous studies, suggesting that ERα is not required to confer receptivity on the rat uterus, nor the present study, showed expression of ERα within a few hours of nidatory E_2 administration, in the P_4 primed ovariectomized uterus, or within the pre-implantation E_2 surge in normal pregnancy (Yoshinaga *et al.,* 1969). Therefore it would be of interest to determine whether the biphasic response of E_2 on ERα regulation in the uterus, when administered alone, also occurs during the window of receptivity to implantation.

The uterus has also been shown to undergo a biphasic change in its receptivity to implantation, within 24 hours, in response to E_2. Prior to the nidatory E_2 dose, in P_4 primed ovariectomized rat uteri, or before day 5 of normal pregnancy, blastocysts are unable to implant into the uterus (Psychoyos, 1973). If blastocysts are transferred into the uterus within 24 hours of E_2 administration in a P_4 primed uterus, or if blastocysts reach the uterus in the afternoon of day 5 of pregnancy

(day 5½), implantation occurs. However after about 36 hours or on day 6½ of pregnancy the blastocysts encounter a hostile uterine environment and are expelled through the vagina (Psychoyos, 1973). It is therefore suggested that, if ERα levels undergo a biphasic change in expression, within 24 hours of the E_2 treatment, in the PPPE regime, it may regulate, in some as yet unelucidated manner, the biphasic change in uterine receptivity to implantation.

Alternatively the biphasic effects of ERα regulation observed in other studies, could be a result of E_2 acting alone, such as during the proliferative phase of the menstrual cycle or during proestrus in rodents.

4.2.3 Changes in ERα Immunolocalization and Changes in Uterine Histology in Response to P_4 and/or E_2 Treatment

4.2.3.1 Histological changes, in response to E_2 treatment, in the luminal epithelium of ovariectomized and pseudopregnant rat uteri, are brought about by the predominant expression of ERα in these cells

In support of possible compartmentalized differences in ERα expression, it is observed that, although a single nidatory dose of E_2 (E) increased ERα expression in all uterine compartments, by fluorescence ICC (Fig.3.4), the most predominant increase was observed in the luminal epithelium. With respect to the PPPE treated rat uteri (Fig.3.10), although ERα immunolocalization did not appear to change between the PPPE (H) and SOOO (F) treated rat uteri, and was present in all three uterine compartments, ERα expression was also predominantly located in the luminal epithelium. In addition, both the single dose E_2 and PPPE treated uteri show results that correlate with previous studies identifying ER (or ERα) localization in hormone treated primate and rodent uteri and in pregnant mice uteri during the peri-implantation period (Hild-Petito *et al.,* 1992; Hiroi *et al.,* 1999; Tan *et al.,* 1999; Wang *et al.,* 1999; 2000; Nephew *et al.,* 2000).

In terms of histological changes (Fig.3.3), E_2 also appeared to have the most dramatic effect on the luminal epithelium of the rat uterus. This was observed by an increase in the height of the luminal epithelial cells and a more convoluted (irregular shaped) lumen, in the single dose E_2 treated rat uteri (Fig.3.3E), compared to the untreated and vehicle treated ovariectomized rat uteri (Fig.3.3A and C). In the PPPE treated H&E sections (Fig.3.9H), the luminal epithelial cell height and luminal convolutions were also increased, compared to the SOOO treated control sections (Fig.3.9F). It can therefore be assumed that at least some of these differences, affecting the luminal epithelium in the PPPE treated rat uteri, arise from the effects of the E_2 treatment. The increase in epithelial cell height and increase in the irregularity of the lumen have been previously suggested to be indicative of E_2's ability to stimulate hypertrophy and hyperplasia, particularly in the luminal epithelium of the rat uterus (Brown *et al.,* 1991; Hosie and Murphy, 1992; 1995; Knobil and Neill, 1994). The histological changes in the luminal epithelium also support the theory that E_2 is predominantly responsible for epithelial proliferation, even though E_2 also stimulates stromal proliferation, whereas P_4 is mainly responsible for stromal proliferation in the uterus (Ljungkvist, 1971b; Clark *et al.,* 1974; Poteat and Bo, 1977; Knobil and Neill, 1994; Hosie and Murphy, 1995). Stromal cell proliferation is also observed in the current study in the E_2 (Fig.3.3E) and PPPE (Fig.3.9H) treated rat uteri, where an increase in uterine diameter is observed.

Luminal epithelial and stromal proliferation have both been observed to occur in the endometrium of humans and animals prior to implantation and have been suggested to be important in providing an appropriate environment for the blastocyst to invade and grow (Finn and Porter, 1975). Prior to implantation there is a small surge of E_2 secretion, which has been suggested to induce the attachment of embryos in the uterus, based on studies in ovariectomized P_4 primed rodents, where the addition of a small amount of E_2, also induces donor embryo implantation (Yoshinaga *et al.,* 1969; Psychoyos, 1973; Finn and Porter, 1975). Therefore E_2's ability to stimulate hypertrophy and hyperplasia of the luminal epithelium may be an important factor in preparing the uterus for this attachment, particularly as the luminal epithelium is the first site of contact between the blastocyst and the maternal uterus (Psychoyos, 1973).

It is therefore interesting that, the luminal epithelium, where the histological effects of E_2 are predominantly observed (Figs.3.3E and 3.9H), is also where there is an increase in ERα expression in the E_2 treated rat uteri (Fig.3.4E), or where ERα is predominantly located in the PPPE treated rat uteri (Fig.3.10H). This suggests that the morphological changes in the luminal epithelium are brought about by E_2 acting through the increased level of ERα that is present in these cells. In other words the predominance of ERα in the luminal epithelium of the rat uterus, when compared to the other uterine compartments, results in the luminal epithelium being more responsive to E_2's stimulatory effect on hypertrophy and hyperplasia, resulting in a columnar luminal epithelium and irregular shaped lumen. The predominant location of ERα in the luminal epithelium at the time of implantation, is also important such that these cells are the first to respond to E_2, in inducing a receptive uterus for implantation.

In the glandular epithelium and stroma, ERα expression is also observed in the E_2 treated (Fig.3.4E) and PPPE treated (Fig.3.10H) rat uteri. However, although in some sections the glandular epithelium also appears to have increased in cell height, the increase is not as obvious as in the luminal epithelium (Fig.3.3E and Fig.3.9H). In addition the expression of ERα does not appear to be as strong in the glandular epithelium, as in the luminal epithelium (Figs.3.4E and 3.10H). This supports the theory that E_2 has differential effects within the different uterine compartments, which has been previously suggested to arise from differences in receptor location, coactivator and/or corepressor expression, expression of other transcriptional activators, and even different growth factor effects, in the different uterine compartments (Poteat and Bo, 1977; Clark and Markaverich, 1982; Clark and Mani, 1994; Dutertre and Smith, 2000). It also might suggest that the extent to which E_2 stimulates hypertrophy and hyperplasia, in a particular uterine compartment, may depend on the level of ERα expression induced by the E_2, in that compartment. In other words, by stimulating a higher expression of ERα in a particular compartment, a greater increase in cell proliferation and cell height occurs.

In contrast to this idea, it was observed that although there was an increase in cell height and proliferation in the luminal epithelium, in the PPPE treated rat uteri (Fig.3.9H) when compared to the vehicle treated rat uteri (F), a similar increase in ERα was not observed in this compartment, by fluorescence ICC (Fig.3.10H). This may suggest that such a link between the level of ERα and cell height and proliferation, does not exist. However, it must be emphasized that neither the single dose (A-E) nor the pregnant and pseudopregnant (F-I) fluorescence ICC results were quantified. Therefore it is suggested that, in future studies, the luminal epithelium, stroma and myometrium, of the uterus, be separated and the expression of ERα be quantified, in each compartment, by Western Blotting and/or RT-PCR.

Alternatively, there may simply be a different expression in certain coregulators, or other transcriptional regulators between the different uterine compartments that result in increases in ERα expression, hypertrophy and hyperplasia in the luminal epithelium, compared to the glandular epithelium, in response to E_2, and that the combination of E_2 and P_4 does not affect the change in hypertrophy and hyperplasia, induced by E_2, but does affect the change in ERα expression.

4.2.3.2 Effect of P_4 on luminal closure in the rat uterus

Another change in the histology of the rat uterus, observed in the current study, is that the luminal surfaces of the PPPE treated and 5½ day pregnant rat uteri (Fig.3.9H and G) are more tightly opposed than in the vehicle (SOOO) treated, non-receptive rat uteri (Fig.3.9F). In addition it appears as though the single dose E_2 treated rat uteri (Fig.3.3E) have a more open or wider lumen profile, than in the untreated (Fig.3.3A) and single dose vehicle treated (Fig.3.3C) rat uteri.

In previous studies, both a single dose and multiple doses of P_4 have been shown to result in similar luminal closure, and when compared to P_4 treated rat uteri, E_2 treated rat uteri have also been observed to have a more open lumen profile (Ljungkvist, 1971a; 1971b; 1971c; Psychoyos, 1973; Knobil and Neill, 1994). However it has been shown that when a subsequent treatment of E_2 is given to P_4 primed ovariectomized rat uteri, the E_2 treatment does not counteract the effects of

P_4 on uterine closure (Knobil and Neill, 1994). Therefore, although a more open lumen may be present in the E_2 treated rat uteri when compared to P_4 treated rat uteri, it is due to the actions of P_4 that result in the luminal surfaces coming into close contact with each other, and not due to the E_2 opening the lumen up.

The P_4 induced uterine closure has been shown to be important for implantation in rats, as it allows the blastocyst to come into close contact with the uterine epithelium, thus aiding in the attachment of the blastocyst to the maternal uterus (Enders and Schlafke, 1967; Ljungkvist, 1971a; 1971b; 1971c; Knobil and Neill, 1994). However it is important to emphasize that the P_4 treatment alone and the resultant uterine closure are not sufficient to induce implantation until a subsequent nidatory dose of E_2 is given (Psychoyos, 1973).

4.2.4 Effect of CC on ERα Expression in the Rat Uterus

4.2.4.1 Clomiphene downregulates ERα levels in the rat uterus

According to the results from the Western blots (Figs.3.1(I) and 3.2(I)) and RT-PCR (Fig.3.6 and Table 3.1) analysis, a single dose of CC (Group D) downregulates the overall expression of ERα, in the rat uterus, 24 hours after CC was administered, compared to the control (Group A [untreated] and Group B [saline treated]) and E_2 treated (Group E) rat uteri. In the pseudopregnant rat uteri, at implantation, although the Western blot (Figs.3.7(I) and 3.8(I)) and fluorescence ICC results (Fig.3.10) showed no significant difference in the overall expression of ERα protein between the untreated (PPPE treated) (H) and CC treated (CCPPPE treated) (Group I) pseudopregnant rat uteri, a slight decrease in the level of ERα protein, is observed in the graph representing the Western blot results (Fig.3.8(I)), in the CCPPPE treated (Group I) rat uteri. In addition CC, given prior to a hormone regime to induce pseudopregnancy (Group I), appeared to downregulate the overall mRNA level of ERα in the rat uterus, compared to the PPPE treated (pseudopregnant) (H), pregnant (G) and vehicle (SOOO) treated (F) rat uteri at the time of implantation (Fig.3.12 and Table 3.2). Therefore, the results from the single dose treatment

groups and pseudopregnant and pregnant treatment groups suggest that CC downregulates ERα expression in the rat uterus at the transcriptional level. However, since such a large decrease is observed in ERα protein following a single dose of CC (D) (Figs.3.1(I) and 3.2(I)), it may be possible that CC also interferes with ERα protein expression at the translational level as well.

Previous studies have also shown reduced ER levels in ovariectomized rat uteri, and in endometrial biopsies taken from postmenopausal women, treated with CC (Kurl and Morris, 1978; Kokko *et al.,* 1981; Brown *et al.,* 1991). However these studies measured the uptake of [^{3}H] estradiol, by the tissue, and therefore did not measure actual ER protein. A similar decrease in ER levels was also found in endometrial biopsies of patients treated with CC, compared to endometrial biopsies of the same patients before treatment, using an enzyme linked immunosorbant assay (ELISA), which does measure true ER protein (Nakamura *et al.,* 1997). The present study is the first study to specifically determine ERα levels in the rat uterus by Western blotting and RT-PCR.

4.2.4.2 Influence of P_4 and/or E_2 on CC's effect on ERα expression

A slight time delay between transcription and translation, may explain to a certain degree, why the decrease in ERα protein levels (Figs.3.7(I) and 3.8(I)) is not yet significantly observed in the CCPPPE treated rat uteri (Group I) at the time of implantation, whereas an obvious decrease is observed in ERα mRNA levels in the same treatment group (Fig.3.12 and Table 3.2), compared to the PPPE treated (H) rat uteri. However, since in the single dose treatments, CC (D) decreased ERα protein expression to such a large degree that undetectable levels of ERα protein were observed (Figs.3.1(I) and 3.2(I)), it is possible that the combination of P_4 and E_2 given to the CCPPPE treated rats, may have also interfered with the effects of CC alone. In other words, the E_2 and P_4 may have counteracted the effects of CC on ERα expression in the rat uterus, such that only a slight decrease is seen in the CCPPPE treated samples (Group I) (Figs.3.7(I) and 3.8(I)), whereas a large decrease in ERα expression is observed in response to CC (D) in the absence of ovarian hormones (Figs.3.1(I) and 3.2(I)).

In support of this theory, it has been shown, with the expression of carbohydrates on the apical surface of the rat uterus, that when CC is administered with P_4, CC acts as a super-estrogen, whereas in combination with E_2, CC acts as an antiestrogen (Hosie *et al.,* 2000). In addition, by administering exogenous P_4 and/or E_2 to women following CC treatment for ovulation induction, the "out of phase" endometrial morphology, decreased endometrial thickness and increased risk of spontaneous abortions, usually observed in CC treated women, has been shown to be improved (Gerli *et al.,* 2000; Elkind-Hirsch *et al.,* 2002). Several investigators have also suggested that there is cross-talk between the ER and PR, and that P_4 can influence expression of genes downstream of the ER (Clemm *et al.,* 1995; Graham and Clarke, 1997; Hewitt and Korach, 2000; Hosie *et al.,* 2000). Since CC binds to and acts via the ER (Clark and Markaverich, 1982), it is possible that both E_2 and P_4 interferes with the effects of CC through cross-talk in their signaling pathways.

Since, in the present study, it was shown that E_2 increases the expression of ERα, the improved affects observed when CC is supplemented with P_4 and/or E_2, mentioned above, may be due to an improved expression of ERα. However in order to confirm this, studies would need to be done, testing the effect of CC alone and in combination with P_4 and/or E_2 on the expression of ERα protein.

4.2.4.3 Decreased ERα could lead to decreased implantation and pregnancy rates, in response to CC

Since it is observed that CC decreases the ERα levels at implantation (Group I), it suggests that this might have an adverse affect on implantation. Even if, as mentioned before, ERα is not a marker for receptivity to implantation, it has been shown to be maintained in the initial stages of pregnancy, until implantation occurs (Hild-Petito *et al.,* 1992). At this point, ERα would be required to prepare the uterus for implantation by interacting with the surge of E_2 that occurs on day 4 of pregnancy, prior to implantation (Yoshinaga *et al.,* 1969), which has been well established to be the important factor in inducing receptivity in a P_4 primed rat uterus for implantation (Yoshinaga and Adams, 1966a; 1966b; Psychoyos, 1973; 1986; Psychoyos and Martel, 1985; Paulson *et al.,* 1990; Paria *et al.,* 1998). The

ERα has also been shown to be the most prominent ER subtype in the uterus (Hiroi *et al.,* 1999; Wang *et al.*, 1999), and therefore is most likely to be the subtype mediating the response to the E_2 that induces implantation. Implantation of donor embryos has also been shown to not occur in ERα knockout mice (Hewitt *et al.,* 2002), thus further supporting a need for ERα to be present in sufficient quantities, in order for implantation to occur.

Since CC is given to women prior to embryo transfer or pregnancy, in order to induce ovulation (Clark and Markaverich, 1982; Plouffe, 2000), it is likely to decrease the level of ERα required to interact with the nidatory surge of E_2 that induces uterine receptivity for implantation. In other words, CC would render the uterus less sensitive to any subsequent estrogen, including the E_2 required to induce implantation. By decreasing the ERα levels, CC may also alter the signaling downstream of ERα, and therefore CC may affect the expression of those E_2 responsive genes involved in preparing a receptive uterus to implantation. In other words by decreasing the level of ERα in the rat uterus in the peri-implantation period, CC may prevent implantation from occurring.

The concentration of E_2 has also been suggested to control the duration of the window of receptivity, where various physiological concentrations of E_2 are able to induce implantation, but lower concentrations keep the implantation window open for longer than higher concentrations (Ma *et al.,* 2003). By reducing ERα in the uterus and hence lowering the sensitivity of the uterus to E_2, CC may also alter when and for how long the window of receptivity is open. In other words, although CC may induce superovulation and allow the ova to become fertilized, when the blastocysts are transferred into or reach the uterus, the window of receptivity may already be closed and the blastocysts will not implant.

An example of where the uterus has a decreased sensitivity to E_2, in response to CC treatment, is observed in the current histological study, when the CCPPPE treated (Group I), PPPE treated (H) and 5½ day pregnant (G) rat uteri are compared (Fig.3.9). In the PPPE treated and pregnant rat uteri, the nuclei of the

luminal epithelium were basally situated, whereas in the CCPPPE treated rat uteri, the nuclei were located in a more central position. Centrally located nuclei have also been observed in the luminal epithelium of CC treated pregnant rat uteri on day 5 of pregnancy (day of implantation), compared to the luminal epithelium of untreated day 5 pregnant rat uteri, which had basally situated nuclei (Poteat, 1981). This change in nuclear location has been shown to be associated with lipid deposition, in the basal region of epithelial cells, which displaces the nuclei into a more central location (Psychoyos, 1973; Poteat, 1981).

Lipid accumulation or the appearance of centrally located nuclei has also previously been shown to occur in the uterine luminal epithelium of rats treated with a single or multiple dose of P_4 and in delayed implantation experiments (Ljungkvist, 1971b; Psychoyos, 1973; Poteat and Bo, 1977; Knobil and Neill, 1994). In the latter experiments, ovariectomized rodents are only primed with P_4 and the blastocysts, transferred into the uteri of these rodents, do not implant, but lie free in the uterine lumen (Psychoyos, 1973). However, if in addition to P_4 treatment, a small dose of E_2 is given, implantation of the donor blastocysts occurs, the lipid droplets disappear from the uterine epithelial cells and the nuclei of these cells occupy a more basal position (Psychoyos, 1973; Knobil and Neill, 1994). Similarly it has been shown that on the morning of day 5 in normal pregnancy (prior to implantation), the nuclei are located more towards the apical plasma membrane in the uterine luminal epithelial cells. However by noon of day 5 (following implantation), the nuclei lie close to the basal membrane (Psychoyos, 1973). Therefore it is proposed that the increase in lipid, and resulting change in nuclear location, observed in these studies is due to the effects of P_4, (not CC) and that E_2, whether given exogenously or as a pre-implantation surge in normal pregnancy, acts to remove this lipid.

With reference to the present study, CC decreases the ERα levels in the rat uterus, such that the uterus is less sensitive to any subsequent E_2 treatment or to the pre-implantation E_2 surge. Therefore the E_2 may not be able to remove the lipid from the luminal epithelial cells, which was induced by P_4 used to prime the uterus for implantation or present during early pregnancy. Thus the lipid would persist in the luminal epithelium of the CCPPPE treated and in the CC treated 5 day pregnant rat

uteri (Poteat, 1981), and result in the appearance of centrally placed nuclei, in these cells. However in the PPPE treated or untreated 5 day pregnant rat uteri (Poteat, 1981), the E_2 treatment or pre-implantation E_2 surge would remove the P_4 induced lipid and so the nuclei are basally situated in the uterine luminal epithelial cells.

This hypothesis would also account for why the nuclei of the luminal epithelial cells, in the single dose CC treated rat uteri, are basally situated in Fig.3.3D, as well as in previous studies, where CC was given to rats in the absence of ovarian hormones (Hosie and Murphy, 1992; 1995). In both the present single dose treatments and in previous studies, there was no P_4 present to induce lipid accumulation in the luminal epithelial cells, thus CC was unable to interfere with the lipid removal effects of E_2, despite decreasing the level of ERα in the rat uteri.

Since the lipid has been shown to be present in pregnant rat uteri prior to implantation and in P_4 primed delayed implantation rat uteri (Psychoyos, 1973; Poteat and Bo, 1977; Knobil and Neil, 1994), it might suggest that the lipid accumulation in the luminal epithelial cells, may play a role in preventing implantation. If this is true, then by decreasing the ERα levels in the rat uterus and decreasing the sensitivity of the rat uterus to E_2, in removing this lipid at implantation, this may be one of the ways in which CC prevents implantation, thus accounting for the low pregnancy rates associated with CC use (Prasad and Kalra, 1967; Poteat, 1981; Birkenfeld *et al.,* 1985; Huet and Dey, 1987; Navot and Laufer, 1989; Nelson *et al.,* 1990; Cohen, 1991; Cummings *et al.,* 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.* 2000). In support of this theory, it has been shown that when CC treated pregnant rat uteri, which had centrally located luminal epithelial cell nuclei on day 5 of pregnancy, were killed on day 8 of pregnancy, no implantation sites were observed. However the normal pregnant rat uteri had basally situated luminal epithelial cell nuclei on day 5 of pregnancy, and normal implantation sites on day 8 of pregnancy (Poteat, 1981).

4.2.4.4 Decreased ERα, in response to CC, affects other estrogen regulated effects in the uterus

Since the decrease in ERα protein in response to a single dose of CC (D) was significantly different to the increase in ERα protein in response to E_2 (E) (Figs.3.1(I) and 3.2(I)), it suggests that CC is antiestrogenic in its effect on ERα regulation.

Many other antiestrogenic effects have also been observed in the uteri of rabbits, rats, mice and humans in previous studies, and have been suggested to affect implantation (Poteat, 1977; Poteat and Bo, 1977; Bowman *et al.,* 1981; Poteat, 1981; Birkenfeld *et al.,* 1986; Dallenbach-Hellweg, 1988; Eden *et al.,* 1989; Brown *et al.,* 1991; Cummings *et al*., 1991; Rogers *et al.,* 1991; Hosie and Murphy, 1992; 1995; Massai *et al*., 1993; Clark and Mani, 1994; Dickey and Holtkamp, 1996; Nakamura *et al.,* 1997; Hosie *et al.,* 1999; 2000; 2003; Biljan *et al.,* 1999; González *et al.,* 2001; Haritha and Rajagopalan, 2003). It is therefore possible that, either CC affects ERα expression and the above E_2 regulated effects separately in the uterus, or that these adverse, antiestrogenic effects occur as a result of the decrease in ERα levels in the uterus, in response to CC treatment. However, in order to confirm this, studies would have to be done, determining whether the same adverse effects occur in the uterus, when reduced levels of ERα are present in the cells. If this hypothesis is true, it might also indicate that a decrease in ERα levels, in the uterus, is one of the first changes that occurs in response to CC treatment. Therefore, if clinicians could increase ERα levels in the uterus, of patients undergoing infertility treatment with CC, without affecting CC's ability to induce superovulation, the adverse effects of CC, on the uterus, and even the implantation and pregnancy rates, of CC treated women, may improve.

In conclusion, by decreasing ERα in the rat uterus, CC may not only render the uterus less sensitive to the effects of the pre-implantation E_2 surge, to induce uterine receptivity, but may also inhibit the expression of many E_2 regulated genes and effects in the uterus, in preparation for implantation. Therefore, this would account for the many adverse antiestrogenic effects observed in the uterus, as well as the low implantation and pregnancy rates observed in rodents and humans, with CC use (Prasad and Kalra, 1967; Poteat, 1981; Birkenfeld *et al.,* 1985; Huet and

Dey, 1987; Navot and Laufer, 1989; Nelson *et al.,* 1990; Cohen, 1991; Cummings *et al.,* 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000).

4.2.4.5 Estrogenic effects of CC in the luminal epithelium

With reference to the histological effects (Figs.3.3D and 3.9I), CC increased the size of the uterus, increased the height of the luminal epithelium and produced a more irregular shaped lumen, compared to the untreated (Fig.3.3A), single and multiple dose vehicle treated (Figs.3.3B and 3.9F) rat uteri. These changes have also been observed in previous histological studies and have been suggested to be due to an increase in hypertrophy and hyperplasia, particularly of the luminal epithelium, in response to CC treatment (Poteat, 1977; Hosie, 1997). Similar effects were also observed, in the current study, in the E_2 treated rat uteri (Fig.3.3E), thus suggesting that CC is estrogenic, with respect to hypertrophy and hyperplasia, particularly in the luminal epithelium.

As mentioned above, the antiestrogenic effects of CC may be due to the overall decrease in ERα levels in response to CC in the uterus. Similarly, it was proposed that the increase in ERα, induced by E_2, could result in an increase in hypertrophy and hyperplasia in the luminal epithelium. It is therefore with interest that, in the fluorescence ICC results (Fig.3.4), there appeared to be an increase in ERα expression in the luminal epithelium with a single dose of CC (D), compared to the untreated and vehicle treated rat uteri (A and B). Therefore it is possible that the increase in ERα in the luminal epithelium might give rise to the estrogenic increases in luminal epithelial cell height and luminal convolutions, whereas the decrease in ERα in the rest of the rat uterus, gives rise to the other antiestrogenic effects observed at the histological and morphological level, in response to CC treatment. In further support of this, E_2 increased the height of the glandular epithelium (Fig.3.3E) and increased ERα immunostaining in the glandular epithelium and stroma (Fig.3.4E), in the present study, whereas CC appeared to decrease ERα expression in the glandular epithelium and stroma (Fig.3.4D) and the glandular epithelium remained cuboidal in shape (Fig.3.3D). Therefore CC is estrogenic in

the luminal epithelium and antiestrogenic in the stroma and glandular epithelium, which supports previous suggestions by Clark and Markaverich, (1982), that CC has both estrogenic and antiestrogenic effects, within the same tissue.

Several studies have shown that there are interactions between the stroma and epithelium in the uterus (Roberts *et al.,* 1988; Cooke *et al.,* 1997). By having an estrogenic effect in the luminal epithelium and an antiestrogenic effect in the stroma and glandular epithelium, CC may adversely affect the coordination between these two compartments, which may adversely affect the preparation of the uterus for implantation. Furthermore, it has been proposed that the arrival of the blastocyst and the preparation of the uterus into a state of receptivity for implantation, is precisely coordinated (Psychoyos, 1973). Therefore even if CC were to induce a receptive luminal epithelium, once the blastocysts start to invade into the underlying stroma, they may encounter an inappropriate environment, and be subsequently be resorbed, as observed in a study in CC treated pregnant rats (Barkai *et al.,* 1992).

It must however be emphasized that the fluorescence ICC results in the present study were not quantified, therefore in order to confirm whether CC affects ERα expression differently in the luminal epithelium, glandular epithelium and stroma, these three uterine compartments should be separated, and the expression of ERα quantified, in each compartment, by Western blotting and/or RT-PCR. This is of particular importance, as there appeared to be no difference in the immunostaining of ERα expression in the luminal epithelium, between the CCPPPE (Group I), PPPE (H) and SOOO (F) treated rat uteri, in the present study (Fig.3.10).

Finally, although the increase in uterine diameter, luminal epithelial cell height and luminal convolutions in response to CC, are estrogenic effects of CC, as observed in the H&E sections (Figs.3.3D and 3.9I) when these effects are compared to the E_2 treated sections (Fig.3.3E), it is observed that CC (D) stimulates these effects, more than what E_2 does (E). In addition the luminal epithelial cells of the CC treated rat uteri (D) appear to be more tightly packed, with a more irregular surface

than in the E_2 treated rat uteri (E). Previous studies have also shown that CC increased the convolutions of the lumen in 5 day pregnant rat uteri, compared to untreated pregnant rats, and that CC increased the cell height of the luminal epithelium, more than what E_2 did (Poteat, 1981; Hosie and Murphy, 1992; 1995). In other words these results suggest that CC has a greater effect on hypertrophy and hyperplasia than E_2, which may also adversely affect endometrial receptivity to implantation.

In the CCPPPE treated rat uteri (Fig.3.9I), the lumen also appeared to be more open than in the PPPE treated rat uteri (H). Although this may also be considered an estrogenic effect of CC on the uterus, it has already been discussed that it is P_4 (not E_2) that determines whether the lumen is open or closed. Therefore it is possible that CC may also interfere with the P_4 signaling pathway, and since uterine closure and P_4 also play a role in implantation (Enders and Schlafke, 1967; Ljungkvist, 1971a; 1971b; 1971c; Psychoyos, 1973; Knobil and Neill, 1994), this interference may also account for the low implantation and pregnancy rates associated with CC use (Prasad and Kalra, 1967; Poteat, 1981; Birkenfeld *et al.,* 1985; Huet and Dey, 1987; Navot and Laufer, 1989; Nelson *et al.,* 1990; Cohen, 1991; Cummings *et al.,* 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000).

4.3 HSP90 EXPRESSION IN THE RAT UTERUS

4.3.1 Effect of Ovarian Hormones on Hsp90 Expression in the Rat Uterus

4.3.1.1 17β Estradiol on Hsp90

Both the Western blot (Figs.3.1(III) and 3.2(II)) and fluorescence ICC (Fig.3.5)results suggest an increase in Hsp90 protein expression in the rat uterus in response to E_2 (E), 24 hours after its administration, compared to the untreated (A), vehicle treated (C) and CC treated (D) rat uteri. In addition this increase in Hsp90 expression was located mostly in the cytoplasm of the luminal epithelium in the E_2 treated rat uteri (E). This supports previous studies in the rat, mouse and sheep uteri where Hsp90 protein and mRNA increased after E_2 treatment, with maximal expression being observed between 18 and 24 hours after E_2 treatment (Ramachandran *et al.,* 1988; Olazabal *et al.,* 1992; Papaconstantinou *et al.,* 2001; 2002). However, although all three previous studies showed that Hsp90 is present in the cytoplasm of all three uterine compartments, the study by Olazabal *et al.,* (1992) indicated that following E_2 treatment Hsp90 immunostaining increased predominantly in the glandular epithelium, whereas the study by Papaconstantinou *et al.,* (2001) showed that Hsp90 immunostaining increased predominantly in the stroma, following E_2 treatment. Both studies contrast with the predominant increase observed in the present study in the luminal epithelium in response to E_2 (Fig.3.5E). The reason for this is not known but may suggest differences in the dosage of E_2 used. The results from the fluorescence ICC analysis were also not quantified and therefore, it is suggested that in future the different compartments of the uterus should be separated and Western blotting performed on each, to determine whether E_2 affects the expression of Hsp90 differently in these different compartments.

4.3.1.2 Effect of P_4 on Hsp90

In the Western blots (Figs.3.7(II); 3.8(II)) and fluorescence ICC analyses (Fig.3.11), no significant difference was found between the Hsp90 levels in the multiple dose vehicle (SOOO) treated (F) and PPPE treated (H) rat uteri at the time of implantation. This suggests that the increase in Hsp90 expression following a single dose of E_2 (E) (Figs.3.1(III), 3.2(II) and 3.5) is counteracted by the P_4 in the PPPE treated rat uteri (H) (Figs.3.7(II); 3.8(II) and 3.11).

In support of this it has been shown that when ovariectomized sheep were treated with P_4 alone, there was no effect on Hsp90 mRNA in the uterus compared to the untreated ovariectomized controls. However when E_2 and P_4 were administered together to ovariectomized sheep, the P_4 treatment decreased the E_2 induced increase in uterine Hsp90 mRNA expression (Wu *et al.,* 1996). This also suggests that there may be cross-talk between the E_2 and P_4 signaling pathways, as has been suggested in previous studies (Clemm *et al.,* 1995; Graham and Clarke, 1997; Hewitt and Korach, 2000).

4.3.2 Hsp90 and ERα

The increase in Hsp90 expression in response to a single dose of E_2 observed in the present study correlated with the increase in ERα expression with the same dose of E_2 in the previous section (4.2.1). There was also no difference in both ERα and Hsp90 expression between the SOOO treated vehicle control (F) and the PPPE treated rat uteri (H) (Figs.3.7(I and II); 3.8(I and II); 3.10 and 3.11). This correlation supports a role for Hsp90 in regulating the transcriptional activity of ERα and in ensuring that the receptor is functional. The similarity in the effect that different hormone treatments have in the expression of these two proteins, might also suggest that there is a link between the level of Hsp90 and the level of ERα in the rat uterus, such that an increase in ERα would also require an increase in Hsp90 to keep the ERα in its most optimal state for the transcription of estrogen responsive genes.

In support of such a link, it has been shown that Hsp90 levels are high in the human endometrium, during the proliferative phase of the menstrual cycle, where serum E_2 and uterine ERα levels are also highest. Similarly in the secretory phase of the menstrual cycle, where E_2 and uterine ERα levels are lowest, Hsp90 levels have also been shown to be low (Yoshinaga *et al.,* 1969; Koshiyama *et al.,* 1995; Wang *et al.,* 2000).

Studies using the specific inhibitors of Hsp90 functioning: geldanamycin and radicicol, have indicated that Hsp90 functions to: 1) maintain ERα in a high

affinity ligand binding conformation (Segnitz and Gehring, 1997; Fliss *et al.,* 2000); 2) prevent degradation of the ERα by the ubiquitin proteasome pathway (Segnitz and Gehring, 1997; Lee *et al.,* 2002); and 3) function in ligand induced transactivation of steroid receptors including ERs (Segnitz and Gehring, 1997; Lee *et al.,* 2002). In addition it has been shown that steroid receptors, such as ERs, are less efficient in cells in the absence of Hsp90 than when Hsp90 is present (Beato *et al.,* 1995). In other words, these studies suggest that ERs cannot function properly in the absence of functional Hsp90 (Beato *et al.,* 1995).

In contrast to this, other studies have suggested that Hsp90 has an inhibitory role on ERα, by keeping the receptor in a non-DNA binding form (Chambraud *et al.,* 1990; Pratt and Toft, 1997). In addition, although studies have suggested that Hsp90 protects the ER from degradation by the ubiquitin proteasome pathway, there is evidence to suggest that this degradation is actually required for transactivation of the ER (Segnitz and Gehring, 1997; Lonard *et al.,* 2000; Lee *et al.,* 2002). Therefore it may be possible that the link between the regulation of ERα and Hsp90, mentioned above, may be more of a balance in the levels of these two proteins, within the uterine cells. In other words it is possible that too high a concentration of Hsp90 may be inhibitory, as it would prevent the necessary transactivational ubiquitin proteasomal degradation of ERα and may even prevent the receptor from binding to the DNA, to induce transcription of E_2 regulated genes. However, too low a concentration of Hsp90 may also be inhibitory, as it may prevent the receptor from binding correctly to its ligand and so prevent the receptor from responding correctly to its ligand, thus preventing the receptor from functioning correctly.

In support of this hypothesis, the presence of Hsp90 has been shown to stimulate the binding of ERs to EREs, within the promoter region of a known E_2 regulated gene *in vitro* (Inano *et al.,* 1994) and therefore Hsp90 was suggested to be a necessary requirement for ER transactivation. However, in another study it was shown that by increasing the concentration of Hsp90 in HeLa cells, the amount of ER-ERE complexes being formed decreased and even preformed ER-ERE

complexes were shown to dissociate (Sabbah *et al.,* 1996). If such a balance in the level of Hsp90 and the level of ERα in a cell exists, then it may explain these two apparently contrasting studies, as well as support the similarity in effect of different hormonal treatments in the expression of ERα and Hsp90 expression in the present study. However further studies would need to be done to determine how Hsp90 can be both stimulatory and inhibitory at different levels in the cell.

4.3.2.1 Hsp90-ERα balance at implantation

The observation that there is no change in Hsp90 expression between the SOOO (non-receptive) (F) and PPPE (H) (receptive) treated rat uteri suggests that there is no change in the expression of Hsp90 at implantation, and therefore Hsp90 is not a marker for receptivity. However, since neither ERα nor Hsp90 levels changed between the non-receptive and (SOOO) and receptive (PPPE) rat uteri, it also indicates that both proteins are at least maintained until implantation occurs. This is particularly important as Hsp90 has been shown to be required for proper ER hormone binding and ER ligand induced transactivation of ERα (Inano *et al.,* 1994; Beato *et al.,* 1995; Fliss *et al.,* 2000; Lee *et al.,* 2002). Therefore it is suggested that the Hsp90 levels are maintained at implantation, to ensure that the receptor is functional, so that the ER can interact with the nidatory E_2 that induces uterine receptivity for implantation.

In conclusion although there is still debate as to whether Hsp90 aids in steroid receptor functioning or inhibits receptor functioning, it is possible that there is a balance between the ERα and Hsp90 levels in the cell, and that by disrupting this balance, either by having too much Hsp90, or too little, the functioning of ERα and other steroid receptors is affected. In addition this balance may play an important role in the preparation of the uterus for implantation, as the correct level of Hsp90 would need to be present to ensure that ERα is functional, so that ERα can induce the transcription of E_2 regulated genes required for implantation to occur.

4.3.3 Effect of CC on Hsp90 Expression in the Rat Uterus

No significant difference was observed in Hsp90 protein levels, in the rat uterus, following a single dose of CC, in the absence of ovarian hormones, compared to the untreated (A) and vehicle treated (B) control uteri, as well as between the CC (D) and E_2 treated (E) rat uteri (Figs.3.1(III) and 3.2(II)). Although a slight decrease in Hsp90 protein expression was observed in the CCPPPE treated rat uteri (Group I), this decrease was also not significantly different to the PPPE treated rat uteri (H) (Figs. 3.7(II) and 3.8(II)). However, although these Western blots may suggest that CC has no effect on Hsp90 expression in the rat uterus, the results did not account for possible differences in Hsp90 expression in the different uterine compartments. In support of a compartmentalized differences in Hsp90 expression with CC treatment, CC (D) increased the staining intensity of Hsp90 protein in the luminal epithelium, compared to the untreated (A) and vehicle treated (B) rat uteri in the fluorescence ICC results (Fig.3.5). Furthermore, Hsp90 protein immunostaining appeared to be less intense in the stroma of the CCPPPE treated (I) rat uteri compared to the PPPE treated (H) rat uteri (Fig.3.11). An increase or decrease in only one of the three uterine compartments may explain why there is only a slight, but non-significant, increase in the Western blot results, with a single dose of CC (D) (Fig.3.2(II)), or why there is only a small, but non-significant, decrease in the CCPPPE treated rat uteri (Group I) (Fig.3.8(II)), compared to the PPPE treated rat uteri (H), as the whole uterus was used in this technique. However, since the ICC results were not quantified, studies separating the epithelium, stroma and myometrium of the uterus and determining the level of Hsp90 protein in each compartment need to be done to confirm whether CC has a differential effect on the expression of Hsp90 in the uterus.

4.3.3.1 Mechanism of CC action on Hsp90 expression in the rat uterus

In terms of the single dose treated rat uteri, Hsp90 levels have been shown to increase in response to E_2 (E), through binding of the ER to a single, but non-consensus ERE within the promoter region of the Hsp90 gene (Koshiyama *et al.,* 1995; Papaconstantinou *et al.,* 2001). Estradiol 17β has also been shown to increase the expression of the heat shock transcription factor, which is known to induce the expression of all heat shock proteins (Hsps) including Hsp90 (Yang *et al.,* 1995). The presence of both the ligand bound ER on the ERE and the heat

shock transcription factor may enhance the level of Hsp90 expression in response to E_2, as observed in the present study.

It has been suggested that one of the reasons why selective estrogen receptor modulators (SERMs), such as CC, can be agonistic in the expression of certain E_2 regulated genes, and antagonistic in the expression of other E_2 regulated genes, within a single cell or tissue type, is that certain EREs within E_2 regulated genes are not recognized by the SERM bound ER, whereas they are recognized by the E_2 bound ER complex. (Dutertre and Smith, 2000). Therefore when CC binds to the ER, it induces a change in conformation of the ER, which may not recognize the non-consensus ERE within the Hsp90 gene. Thus CC would not induce the expression of Hsp90 and so the levels of Hsp90 protein (D) remain the same in the rat uterus, compared to the untreated (A) and vehicle treated (B) control rat uteri, as observed in the present study. However the E_2 bound ER would recognize the Hsp90 ERE and so the levels of Hsp90 increase in the rat uterus, following E_2 treatment.

It has also been suggested that SERMs can act as an antagonist in one cell type and as an agonist in another cell type, due to localized differences in the expression of coactivators, corepressors and/or other transcription factors within cells (Dutertre and Smith, 2000; Hall *et al.,* 2001). It is possible that the expression of the heat shock transcription factor is only localized to the luminal epithelium in the rat uterus. If CC is able to induce the expression of this transcription factor, it would increase the expression of Hsp90 only in the luminal epithelium, as observed in the fluorescence ICC results (Fig.3.5). Further studies identifying the effect of CC on the heat shock transcription factor, would therefore need to be done in order to confirm this hypothesis. Alternatively the luminal epithelium may contain coregulators or transcription factors (other than the heat shock transcription factor) that would allow CC to increase Hsp90 levels in the luminal epithelium, whilst not affecting Hsp90 expression in the rest of the rat uterus.

With respect to the slight, but non-significant decrease in Hsp90 levels observed in the CCPPPE treated rat uteri (Group I), compared to the PPPE treated rat uteri (H) (Figs.3.7(II); 3.8(II) and 3.11), it is possible that the decrease is due to the effects

of P_4 and/or E_2 on the effects of CC. Similar hormonal interference on CC induced changes in the expression of surface carbohydrates on the luminal epithelium of the rat uterus have also been observed (Hosie *et al.,* 1999; 2000). Progesterone has also been shown to decrease the E_2 induced increase in Hsp90 levels in ovariectomized sheep endometrium, when E_2 and P_4 were administered together (Wu *et al.,* 1996). It is therefore probable that when CC is administered with either E_2 or P_4, or both, that it results in a decrease in Hsp90 expression in the rat uterus, possibly through cross talk between the signaling pathways of E_2 and P_4.

It is however clear that more studies, analysing the effects of CC in combination with P_4 and/or E_2, and the effects of CC on the signaling pathways of E_2 and P_4, need to be done in order to elucidate the mechanism of action of CC on Hsp90 levels.

4.3.3.2 Influence of CC on ERα functioning through changes in Hsp90 expression

Despite the possibility of localized differences in Hsp90 expression in response to CC, it appears that CC does not influence the overall expression of Hsp90 in the rat uterus, whether in the absence of ovarian hormones (D) or at implantation (I). However, CC does decrease the overall expression of ERα in the rat uterus (section 4.2.4). A decrease in ERα levels, but no change in Hsp90 levels, in response to CC, may upset the proposed balance in the levels of these two proteins, which may be required to ensure proper ERα functioning, by maintaining its transactivational and ligand binding properties (Beato *et al.,* 1995; Segnitz and Gehring, 1997; Fliss *et al.,* 2000; Lee *et al.,* 2002). In other words, CC may result in the level of Hsp90 being too high, which may be inhibitory on the signaling of ERα, by preventing the necessary degradation of the receptor, as well as preventing receptor dimerization and DNA binding (Chambraud *et al.,* 1990; Pratt and Toft, 1997; Segnitz and Gehring, 1997; Lonard *et al.,* 2000; Lee *et al.,* 2002). By preventing proper ERα functioning, the expression of a number of E_2 regulated genes may therefore be inhibited, in response to any subsequent E_2, in the rat uterus. In addition since E_2 has been shown to be essential for inducing a receptive uterus for implantation (Yoshinaga and Adams, 1966a; 1966b; Psychoyos, 1973; Paria *et al.,* 1998), the effect of CC on ERα functioning may also adversely affect

implantation, thus giving rise to the observed low implantation and pregnancy rates in CC treated rodents and women (Navot and Laufer, 1989; Cohen, 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000).

It must however be mentioned that Hsp90 not only binds to and influences the functioning of ERα, but also binds to other steroid receptors, including PRs, present in the cell (Beato *et al.,* 1995; Pratt and Toft, 1997). Therefore it may be difficult to interpret the effect of CC on ERα functioning, with respect to CC's effect on Hsp90 expression, without determining the effect of CC on the expression of other steroid receptors as well.

4.3.4 Conclusion to CC's Effect on ERα and Hsp90 Expression in the Rat Uterus

In conclusion CC affects the expression of genes involved in the estrogen signaling pathway in the rat uterus both in the absence of hormones and at implantation, by downregulating the receptor, ERα. In addition, even though CC may not affect Hsp90 expression to an appreciable degree, and only affects ERα expression, this may also impair ER signaling, by disrupting the balance between the ERα and Hsp90 levels in the rat uterus.

The effect of CC on ERα and Hsp90 is also observed to be an antiestrogenic effect, as it is opposite to the effects of E_2 on the expression of these two genes in the rat uterus. Since E_2, through its receptor, ERα, plays an important role in inducing receptivity in the uterus for implantation (Yoshinaga and Adams, 1966a; 1966b; Psychoyos, 1973; Paria *et al.,* 1998), CC would adversely affect implantation by reducing the sensitivity of the uterus to any subsequent E_2, including the nidatory E_2. These affects would therefore explain many of the adverse antiestrogenic effects that have been observed with CC treatment in the uterus (Bowman *et al.,* 1981; Hosie and Murphy, 1992; 1995; Hosie *et al.,* 1999). It would also explain the low implantation and pregnancy rates associated with CC use, despite its efficiency at inducing ovulation (Clark and Markaverich, 1982; Navot and Laufer, 1989; Cohen, 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000).

4.4 HOXA10 EXPRESSION IN THE RAT UTERUS

No distinct results were obtained in the present study as to the effect of CC on Hoxa10 expression, particularly with Western blotting (Fig.3.13). Reasons for why these blots did not work may suggest that Hoxa10 protein expression was too low in the uterus for the antibodies to detect or that the antibodies were not of high enough quality to detect Hoxa10 in the uterus.

It is felt that the latter reason is more likely as 35µg of protein was loaded onto the gel, which also corresponded to the maximum volume of the lowest concentrated sample that could be loaded onto the gel. To load higher concentrations of protein, the samples would have had to been dialyzed or re-extracted with a smaller volume of homogenizing buffer. At such high concentrations, the proteins might not have separated properly and the samples would have smeared. A high concentration of primary antibody was also used (1:50), so it is unlikely that the amount of antibody used was insufficient to detect the protein. Since actin was also correctly detected on the blots, it suggests that there were no faults in the separation of the proteins or in the transfer procedure.

The negative controls for the fluorescence ICC results, in Fig.3.14 (NegI and II) for Hoxa10 expression, showed only autofluorescing red blood cells, therefore it can be seen that the antibodies for Hoxa10 used in this procedure were specific. The primary antibody used in the fluorescence ICC analysis, was the same as that used for the Western blots. This suggests that the problem with the Western blots may lie with the secondary antibody, and therefore it is suggested that in future a different secondary antibody should be used for the detection of Hoxa10 in Western blotting.

It is also important to note that since the negative control sections for Hoxa10 expression, in the fluorescence ICC results (Fig.3.14), were serial to each other, the fluorescence observed in these sections does represent autofluorescing red blood cells and not dirt or antibody precipitate, as the fluorescence is in the same position in both sections (RBC and arrows).

4.4.1 Effect of a Nidatory Dose of E_2 on Hoxa10 Expression in the Rat Uterus

In the present study there appears to be no difference in the staining intensity or location of Hoxa10 protein following a single dose of E_2 (Fig.3.14E), compared to the oil treated vehicle control (Fig.3.14C). This result is somewhat surprising as previous studies have shown that the human Hoxa10 gene, which is said to be highly conserved, contains an ERE that is able to bind to both ERα and ERβ (Krumlauf, 1994; Akbas *et al.,* 2004). In addition, when this ERE was transfected, with a luciferase expression construct, into Ishikawa cells, it was shown to drive E_2 induced luciferase reporter expression (Akbas *et al.,* 2004). Vascular permeability, which has been shown to be induced by E_2, has also been shown to be decreased in homozygous Hoxa10 mutant (Hoxa10 -/-) mice uteri, on day 4.5 of pregnancy (Psychoyos, 1973; Benson *et al.,* 1996; Ma *et al.,* 1998; Rockwell *et al.,* 2002).

Other studies have also shown that E_2 can induce changes in Hoxa10 expression in the uterus. In the study by Ma *et al.,* (1998), E_2 decreased Hoxa10 expression, whereas in other studies E_2 increased Hoxa10 expression in the uterus (Taylor *et al.,* 1998; Gui *et al.,* 1999). However, both the increase and decrease in Hoxa10 expression, in response to E_2 treatment, was shown to be unaffected by the protein synthesis inhibitor, cycloheximide (Ma *et al.,* 1998; Taylor *et al.,* 1998). This was suggested to indicate that Hoxa10 expression is a direct target of the ER (Ma *et al.,* 1998; Taylor *et al.,* 1998), therefore indicating that E_2 does regulate Hoxa10 expression in the uterus, thus contrasting with the results of the present study.

It is possible that the apparent contrasting results of the effect of E_2 on Hoxa10 expression, might reflect differences in the dose of E_2 used in the above mentioned studies. It is therefore possible that a higher dose of E_2, such as that found in the proliferative (proestrus) phase of the menstrual (estrus) cycle, or during ovulation (Yoshinaga *et al.,* 1969; Psychoyos, 1973; Finn and Porter, 1975), would decrease the level of Hoxa10 expression in the uterus. However smaller doses, such as the nidatory E_2 used to induce receptivity in the ovariectomized P_4 primed uterus, or the pre-implantation E_2 surge (Yoshinaga *et al.,* 1969; Psychoyos, 1973), might increase Hoxa10 expression in the uterus. In support of such a hypothesis, it has been shown that Hoxa10 expression is low during the proliferative phase of the human menstrual

cycle, when E_2 levels are high, and on day 0.5 of pregnancy in mice uteri, following the ovulatory pulse of E_2 (Satokata *et al.,* 1995; Taylor *et al.,* 1998; Gui *et al.,* 1999). Furthermore, a larger dose of E_2 (250ng) was used to treat the mice, in the study where E_2 decreased Hoxa10 expression, instead of the small (25-100ng) E_2 dose usually used to induce implantation in ovariectomized P_4 primed mice uteri (Ma *et al.,* 1998; Reese *et al.,* 2001).

It would therefore be expected that the single nidatory dose of E_2, used in the present study (E), would increase the expression of Hoxa10 in the rat uterus. However, since only a small (2 fold) increase in Hoxa10 levels was observed in the human endometrium, in response to E_2 treatment (Taylor *et al.,* 1998), it is possible that the increase in Hoxa10 levels might not be great enough to be visually observed in the fluorescence ICC results of the present study. Therefore this would account for the apparent similarity in Hoxa10 expression in the present study between the E_2 treated (E) and untreated (A) or vehicle treated (C) ovariectomized rat uteri. It also emphasizes the need to quantify the results for Hoxa10 expression in the rat uterus by Western blotting or RT-PCR.

4.4.2 Effect of P_4 on Hoxa10 Expression Levels in the Rat Uterus

There appears to be an slight increase in the stromal expression of Hoxa10 in the fluorescence ICC results (Fig.3.15) of the PPPE treated (receptive) (H) rat uteri, compared to the SOOO treated (non-receptive) (F) rat uteri. This corresponds with previous studies indicating peak expression of Hoxa10 expression in the uterus during the mid-secretory phase of the menstrual cycle in human, and on day 3.5 of pregnancy in mice, both of which represent stages when the uterus is most receptive to implantation (Satokata *et al.,* 1995; Taylor *et al.,* 1998). Therefore the results of the present study appear to support a role for Hoxa10 expression in determining receptivity to implantation in the uterus.

The increase in Hoxa10 expression in the PPPE treated rat uteri (H), in the current study, also supports the hormonal regulation of this gene in the uterus, as observed in other studies. In these previous studies, it was shown that maximal Hoxa10

expression was induced in human uterine stromal cells, when P_4 and E_2 were administered together (Taylor *et al.,* 1998). In addition in ovariectomized mice uteri, the combination of E_2 and P_4 showed a restricted pattern of Hoxa10 expression to the antimesometrial part of the uterus (Ma *et al.,* 1998), which is where implantation is known to occur in both mice and rat uteri (Enders and Schlafke, 1967; Psychoyos, 1973; Carson *et al.,* 2002).

Due to the observations in this study that E_2 did not affect the expression of Hoxa10 in the rat uterus (Fig.3.14E), it is also possible that the increase in Hoxa10 expression in the PPPE treated rat uterus (Fig.3.15H), is due to the effects of P_4 and not due to the combination of P_4 and E_2. In support of this it has been shown that P_4 increased Hoxa10 expression in the stroma of ovariectomized mice uteri, whereas in the same study E_2 decreased Hoxa10 expression (Ma *et al.,* 1998). Progesterone also increased Hoxa10 expression in human endometrial stromal and epithelial cells and in the Ishikawa cell line by as much as 25 fold, whereas the increase by E_2 in the same study was only 2 fold (Taylor *et al.,* 1998; Gui *et al.,* 1999). This increase in response to P_4 was also shown to be directly mediated by the PR, as it was not affected by cycloheximide, but was inhibited by a PR specific inhibitor, RU486 (Ma *et al.,* 1998). Therefore P_4, and not E_2, appears to be the major hormone responsible for Hoxa10 expression in the uterus.

Hoxa10 also appears to be more important in mediating P_4 regulated gene expression rather than regulating E_2 gene expression. This is due to previous studies that have shown uterine changes in the decidualization response, stromal cell proliferation, prostaglandin E2 receptor subtype expression, number of implantation sites and embryo survival, pinopod expression, cyclin dependent kinase inhibitor expression, T cell lymphocyte proliferation, T cell specific gene expression and Bip (calcium binding Hsp70 chaperone protein) expression, either in response to changes in uterine Hoxa10 expression or between Hoxa10 -/- mutant and wildtype mice uteri (Satokata *et al*., 1995; Benson *et al*., 1996; Taylor *et al*., 1998; Lim *et al*., 1999; Bagot *et al.,* 2000; Bagot *et al.,* 2001; Yao *et al.,* 2003). All these effects have been previously shown to involve, or be regulated by, P_4 and many of these effects have been linked to receptivity to implantation in the uterus (Finn and Porter,

1975; Martel *et al.*, 1991; Knobil and Neill, 1994; Lim *et al.,* 1999; Graham and Clarke, 1997; Hewitt and Korach, 2000; Reese *et al.,* 2001; Yao *et al.,* 2003). Therefore in all these studies, including the present one, it appears as if Hoxa10 expression may be more closely linked to mediating P_4 gene regulation, rather than E_2 gene expression. In addition it is possible that it is P_4 that controls the expression of Hoxa10 in the rat uterus, rather than E_2, or a combination of both.

4.4.3 Effect of Ovarian Hormones on the Location of Hoxa10 in the Rat Uterus

In the fluorescence ICC analysis (Fig.3.14 and Fig.3.15), expression of Hoxa10 appears to be located within the nuclei of the luminal epithelium, glandular epithelium and stroma, in all treatment groups (A-I). Epithelial expression of Hoxa10 has also been observed during the menstrual cycle of humans and during early pregnancy in adult mice (Satokata *et al.,* 1995; Taylor *et al.,* 1997; 1998; Gui *et al.,* 1999). However in the study by Ma *et al.,* (1998), the epithelial expression of Hoxa10 in mice uteri was not affected by either P_4 or E_2, compared to ovariectomized oil treated controls. This study suggested that either the concentration of steroid hormones used in that study was not sufficient to induce Hoxa10 expression in the epithelium, or that epithelial Hoxa10 expression is steroid independent (Ma *et al.,* 1998). Expression has also been observed in isolated endometrial cells grown in culture from human endometrial biopsies as well as in a uterine adenocarcinoma cell lines (Ishikawa cell line), in the absence of steroid treatment (Taylor *et al.,* 1998; Gui *et al.,* 1999). Therefore it is likely that expression of Hoxa10 expression is present in the epithelium independent of steroid hormones. Since it was observed that E_2 and CC did not change the expression of Hoxa10 in the present study, it makes sense that the location of Hoxa10 in the E_2 (E) and CC (D) treated sections is in the epithelium (Fig.3.14).

The predominant expression of Hoxa10 in the stroma of the pregnant (G) and PPPE treated (H) rat uteri (Fig.3.15) supports the studies in mice uteri, where expression of Hoxa10 was found only in the stroma (Ma *et al.,* 1998) following P_4 treatment. However in the single dose treatments (Fig.3.14), that is, in the saline

(B), oil (C), CC (D) and E_2 (E) treated sections as well as in the SOOO treated (F) sections (Fig.3.15) Hoxa10 is observed predominantly in the luminal and glandular epithelium. It is therefore suggested that P_4 upregulates Hoxa10 protein expression in the stroma and possibly downregulates it in the luminal and glandular epithelium. To confirm this it is suggested that future studies should be done separating the different compartments and the effect of E_2 and/or P_4 be tested on Hoxa10 expression in the whole uterus as well as in the different compartments.

Further support for the steroid independent expression of Hoxa10 in the luminal epithelium and the stromal regulation of Hoxa10 by P_4, can be seen in pregnant mice (Satokata *et al.,* 1995). In that study, it was shown that on days 0.5-1.5 of pregnancy in the mouse uterus, following the ovulatory surge in E_2 levels, Hoxa10 mRNA expression was strongly expressed in the luminal and glandular epithelium. However by day 3.5 of pregnancy (pre-implantation stage) when P_4 levels rise, Hoxa10 mRNA was detected in the stroma underlying the epithelium. Then between days 4.5 - 7.5 of pregnancy, expression of Hoxa10 continued to be strong in the developing decidua, which consists of specialized stromal cells (Satokata *et al.,* 1995). Strong stromal expression of Hoxa10 has also been shown in the human pregnant decidua (Taylor *et al.,* 1998). Hoxa10 mRNA and protein expression has also been shown to peak at the mid-secretory phase of the menstrual cycle in human endometrium (Taylor *et al.,* 1998; Gui *et al.,* 1999), which in addition to corresponding to the most receptive state of the uterus during the menstrual cycle, also corresponds to a period when P_4 levels begin to rise (Yoshinaga *et al.,* 1969; Finn and Porter, 1975). Hoxa10 levels also remain elevated throughout the secretory phase, whilst low levels of Hoxa10 expression are present in the proliferative phase (Taylor *et al.,* 1998; Gui *et al.,* 1999).

Progesterone levels have been shown to be high during the secretory phase of the menstrual cycle and during pregnancy, and P_4 has also been shown to be particularly important in decidualization (Yoshinaga *et al.,* 1969; Psychoyos, 1973; 1976; Finn and Porter, 1975). This further supports a potential for P_4 in regulating stromal Hoxa10. Since P_4 also plays an important role in implantation (Psychoyos, 1973; 1976), the stromal upregulation of Hoxa10 in the uterus, in response to P_4, may play a more important role in regulating the expression of genes involved in

the differentiation of the stroma, for the later invasion of the embryo into the underlying stroma and in decidualization.

4.4.4 Effect of CC on Hoxa10 Expression in the Rat Uterus

With respect to the CC treated sections in the present study, there appears to be no difference in staining intensity or location of Hoxa10 expression in the single dose CC treated sections in Fig.3.14D, compared to its vehicle control (Fig.3.14B) or to the E_2 treated sections (Fig.3.14E). There also appears to be no difference in expression of Hoxa10 between the CCPPPE treated sections (Fig.3.15I) and the PPPE treated sections (Fig.3.15H). Therefore it appears that CC does not alter the expression of Hoxa10 expression in the rat uterus following a single dose of CC in the ovariectomized rat, or at the time of implantation.

In light of the information suggesting that P_4 is the predominant hormone affecting Hoxa10 expression, and that E_2 has either no effect or only a minimal effect on Hoxa10 expression, it is perhaps not that surprising that CC was observed to have no effect on Hoxa10 expression. This is due to observations that CC binds to, and acts through, the ER, and therefore influences the E_2 signaling pathway, despite the possibility of cross-talk between the ER and PR signaling pathways (Clark and Markaverich, 1982; Clemm *et al.,* 1995; Graham and Clarke, 1997). In support of this, no difference in Hoxa10 expression was shown when the Ishikawa (endometrial adenocarcinoma) cell line was treated with CC (Cermik *et al.,* 2003). However it is debatable as to whether such a cell line would represent the true regulation of Hoxa10 in the uterus, particularly if CC only has an affect on stromal Hoxa10 and not on epithelial Hoxa10. It must therefore be emphasized that the fluorescence ICC results were not quantified and it is also possible that CC may only affect Hoxa10 expression in one of the uterine compartments. Therefore if the results were quantified and the individual uterine compartments separated, a slight difference in Hoxa10 expression, in response to CC treatment, may be observed.

In support of a possible decrease in Hoxa10 expression in response to CC, it has been shown that CC decreases the expression of β_3 integrins, in the uterine

epithelium, and increases β_3 integrin expression in the human endometrial stroma, at the time of implantation, compared to normal fertile control endometrial expression (González *et al.,* 2001). Hoxa10 expression has been shown to directly regulate β_3 integrins in the human endometrium. This was due to the observation that β_3 integrin mRNA levels decreased in human endometrial samples shown to have low Hoxa10 expression, and in Ishikawa cells transfected with Hoxa10 antisense oligonucleotides, whereas when Hoxa10 was constitutively expressed in Ishikawa cells, β_3 integrin mRNA increased (Daftary *et al.,* 2002). Since Hoxa10 regulates these integrins, then a possible aberrant expression in Hoxa10 by CC may mediate the adverse affects on β_3 integrins observed in patients treated with CC. The β_3 and α_4 integrins have been shown to act as markers of receptivity and they have been thought to aid in the attachment of the embryo to the maternal uterus (Lessey *et al.,* 1992; Carson *et al.,* 2000; Lessey, 2003). In addition the $\alpha_4\beta_1$ integrins have been shown to be upregulated by a nidatory dose of E_2 in a P_4 primed mouse uterus (Basak *et al.,* 2002). Therefore a possible adverse effect on Hoxa10 expression and subsequent integrin expression, may give rise to the observed low implantation and pregnancy rates associated with CC treatment in both rodents and humans (Prasad and Kalra, 1967; Poteat, 1981; Birkenfeld *et al.,* 1985; Huet and Dey, 1987; Navot and Laufer, 1989; Nelson *et al.,* 1990; Cohen, 1991; Cummings *et al.,* 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000).

In conclusion the predominant steroid affecting Hoxa10 expression is P_4, and since CC appears to mainly affect E_2 regulated genes, including the ERα, it is possible that CC does not regulate Hoxa10. Therefore the adverse affects on implantation and pregnancy rates observed with CC are more likely to be mediated by adverse affects on genes regulated by the pre-implantation surge in E_2 than in genes regulated by P_4. However, since the results in the present study were not quantified, it is still possible for a slight or even localized response in Hoxa10 expression, following CC treatment, in the rat uterus. It is also concluded that epithelial expression of Hoxa10 appears to be independent of steroid hormones, whereas stromal expression is regulated by P_4. Much research is however still required in order to confirm either hypothesis.

4.5 FUTURE DIRECTIONS FOR THIS STUDY

1. Although, a broad range of E_2 doses has been used in studies to induce donor embryos to implant in ovariectomized P_4 primed rats, a higher implantation success rate has been shown to occur with lower doses (such as 0.1μg), and can also lengthen the window of receptivity in the uterus (Psychoyos, 1961; 1973; Ljungkvist, 1972; Hammer *et al.,* 1978; Ma *et al.*, 2003). It is suggested that in future studies a lower dose of E_2, such as 0.1μg, should be used to induce implantation in ovariectomized P_4 primed uteri, which may produce a receptive uterus that resembles the 5½ day pregnant rat uteri more closely.

2. In order to elucidate the effects of CC on gene expression during implantation more clearly, it is suggested that gene expression in CCPPPE treated rat uteri, be compared to the effects of CC alone as well as in combination with E_2 or P_4, in ovariectomized rat uteri. This will aid in determining how CC acts at the gene level and how the ovarian hormones influence CC's effect on gene expression.

3. To quantify the compartmentalized differences in gene expression with CC treatment, in the rat uterus, it is suggested that the luminal epithelium, glandular epithelium, stroma and myometrium be separated. The expression of genes should then be quantified in each compartment, by Western blotting and/or RT-PCR. This may also account for slight increases or decreases in the overall expression of Hsp90 and ERα in the rat uterus following CC treatment.

4. Since the uterus is known to undergo a biphasic change in its receptivity to implantation, it would be of interest to determined whether a biphasic change in ERα expression occurs during the window of receptivity in the uterus, as occurs when E_2 is administered alone to ovariectomized rats (Psychoyos, 1973; Rosser *et al.,* 1993; Zhou *et al.,* 1993; Nephew *et al.,* 2000). This could be achieved by determining the expression of ERα before and at four, six, 12, 18, 24 and even 36 hours after the E_2 surge on day 4 of pregnancy in rats, or after the E_2 treatment in the PPPE treated rat uterus.

5. In terms of quantifying the effects of CC on Hoxa10 expression in the rat uterus, it is suggested that Western blotting, using a different source of antibodies, or RT-PCR analysis, with Hoxa10 specific primers, should be done.

6. Finally, the effects of CC on implantation at the gene expression level is still far from being understood. It is therefore suggested that changes in the expression of other implantation specific genes be assessed with CC treatment, particularly those that are influenced by the nidatory dose of E_2, that induces receptivity in the uterus for implantation.