CHAPTER 1: INTRODUCTION

1.1 CLOMIPHENE CITRATE

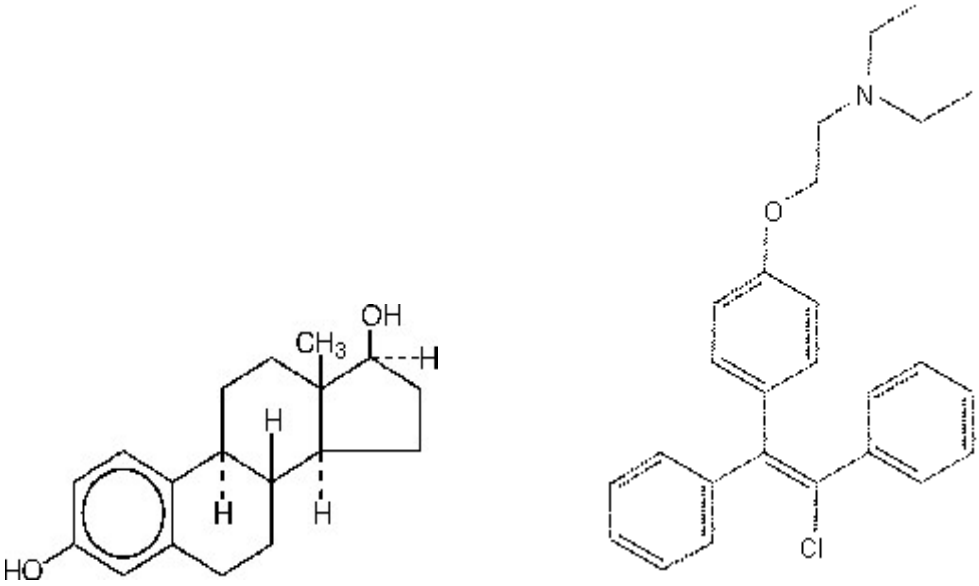
Clomiphene citrate (CC) is the most prescribed drug used in infertility treatment (Edwards *et al.*, 1996; Plouffe, 2000). It will induce ovulation in 70-80% of women using the drug. It also has few long term side effects, is relatively cheap to produce, and is simple to administer (Cohen, 1991; Derman and Adashi, 1994; Edwards *et al.*, 1996). Clomiphene is therefore the first prescribed drug used in assisted reproductive procedures, such as, *in vitro* fertilization (IVF) and gamete intra-fallopian tube transfer (GIFT), and often replaces gonadotropin therapy, which is occasionally hazardous and difficult to control (Lunan and Klopper, 1975; Derman and Adashi, 1994; Dickey and Holtkamp, 1996; Hughes *et al.,* 2000).

Clomiphene is given to women as a 38:62% racemic mixture of its *cis* (zuclomiphene) and *trans* (enclomiphene) isomers (Dickey and Holtkamp, 1996). It is usually administered as an oral daily dose of 50mg for five days starting on the 5^{th} day of the menstrual cycle following spontaneous or progesterone (P_4) induced menstruation. Ovulation then occurs approximately 7 days after CC treatment (Clark and Markaverich, 1982). To induce ovulation CC acts as a competitive antagonist of 17β estradiol (E_2) in the hypothalamic arcuate nucleus, thus alleviating the negative feedback effects of E_2 and resulting in an increase in gonadotropin-releasing hormone (GnRH) concentration. Clomiphene also increases the sensitivity of the pituitary and ovary to GnRH, which results in an increase in follicle stimulating hormone (FSH) secretion by the pituitary, which in turn induces ovulation (Dickey and Holtkamp, 1996).

In spite of CC being an extremely efficient superovulator, the problem with CC is that the pregnancy rates resulting from its use are very low, even when used in normal ovulatory women (Navot and Laufer, 1989; Cohen, 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997), with pregnancy rates as low as 2.5% per cycle been reported (Hughes *et al.,* 2000).

1.1.1 Clomiphene as a Selective Estrogen Receptor Modulator (SERM), and the Interaction of SERMs with the Estrogen Receptor (ER)

Clomiphene Citrate is a synthetic non-steroidal estrogen agonist-antagonist that belongs to the triphenylethylene family (Clark and Markaverich, 1982; Jordan, 1984). Triphenylethylenes, including CC, show structural similarities to E_2 (fig.1.1) and are known to bind to both estrogen receptors alpha (ERα) and beta (ERβ), thus influencing the receptors' gene transcription behavior and ultimately E_2's physiological and structural effect within the target organ (Clark and Markaverich, 1982; Jordan, 1984; Pike *et al.,* 1999). Compounds, like CC, which bind to the estrogen receptors (ERs) and which have such mixed agonistic and antagonistic effects, are known as selective estrogen receptor modulators (SERMs) (Jordan *et al.,* 2001).



A) 17β Estradiol **B) Clomiphene Citrate**

Fig.1.1 Molecular Structure of 17β Estradiol (estrogen) (A) (Novartis, 2004) and Clomiphene citrate (CC) (B) (adapted from Spirov, 2003)

The ability of the SERMs to exhibit agonistic or antagonistic effects on the estrogen signaling pathway within a single tissue or cell type is thought, at least partly, to be due to the binding dynamics of these molecules to the ER's ligand binding domain (LBD) (Brzozowski *et al.,* 1997; Shiau *et al.,* 1998; Pike *et al.,* 1999; 2000; 2001; Hubbard *et al.,* 2000; Kong *et al.,* 2003).

Most of the studies on the binding of different SERMs, pure agonists and antagonists to the ER LBD, have been carried out on E_2 (Brzozowski *et al.,* 1997), tamoxifen (Shiau *et al.,* 1998), raloxifene (Brzozowski *et al.,* 1997; Pike *et al.,* 1999), diethylstilbestrol (Shiau *et al.,* 1998), genistein (Pike *et al.,* 1999) and ICI 164,384 (Pike *et al.,* 2001). The size and shape of the ER's ligand binding cavity, as well as the plasticity of the components within the LBD, allow different structural motifs to bind to the ER, therefore allowing a wide range of molecules to bind to it (Brzozowski *et al.,* 1997; Shiau *et al.,* 1998; Pike *et al.,* 1999). Each ligand will interact with specific residues within the LBD, resulting in a distinct orientation of the activation function (AF)-1 and AF-2 domains, which are the domains responsible for transactivation of the receptor. In addition the AF-2 region lies within the LBD and is therefore responsible for ligand induced transactivation (Pike *et al.,* 1999; 2000; Hubbard *et al.,* 2000; Kong *et al.,* 2003).

Within the structure of the ER, there are 12 alpha helices: H1-H12. When pure agonists such as E_2 are bound to the ER, H12, which contains the core of the AF-2 transactivation region, completely covers E_2 within the LBD, thus burying it in the hydrophobic core of the ER protein. This event also triggers changes in the conformation of the AF-1 domain, and together these changes allow the ER to recruit and interact with components of the transcriptional machinery, as well as with coregulators (coactivators and corepressors), thereby facilitating or suppressing gene expression (Pike *et al.,* 2000; 2001; Kong *et al.,* 2003). However, with partial agonists such as genistein; SERMs such as raloxifene and tamoxifen; and full antagonists, such as ICI 164,384, the H12 helix is displaced, and the ligand is therefore not completely covered by this AF-2 containing region. It also appears that the more displaced the H12 region is, the more antagonistic the function of that ligand (Pike *et al.,* 2000; 2001; Kong *et al.,* 2003).

When raloxifene binds to the ER, the bulky side chain of raloxifene protrudes from the LBD of the ER. Therefore, H12 is displaced to a shallow groove formed by H3 and H5, thus preventing the proper formation of AF-2, and consequently raloxifene induces antagonism (Kong *et al.,* 2003). On the other hand when genistein, which is a partial agonist similar in size to E_2, is bound to the ER the orientation of H12 results in only a 'quasi'-antagonist position (Pike *et al.,* 1999; 2000; Kong *et al.,* 2003). The binding of coactivators has been shown to be highly dependent on the orientation of this H12 region of the receptor (Pike *et al.,* 2000). Therefore, by displacing the positioning of H12, a different receptor conformation is achieved, to that induced by E_2. This new conformation of the ligand-ER complex is thought to be unable to recruit the necessary coactivators and may even prevent the interaction of the ligand bound ER with other transcription factors, required to fully activate the transcription of certain estrogen responsive genes. In other words the differences in receptor conformation, induced by these different SERMs, results in a different pool of coregulators and/or transcription factors being recruited to the transcriptional machinery, which differ from those that are recruited by E_2 binding. Therefore the transcriptional response of the ER is altered, resulting in either partial agonistic or antagonistic effects in a particular tissue (Pike *et al.,* 1999; 2000; Kong *et al.,* 2003). When the pure antagonist ICI 164,384 binds to the ER, its bulky side chain completely destabilizes the positioning of H12, such that it neither lies in the groove formed by H3 and H5, nor does it cover the ligand in the LBD. This conformation of the ligand-ER complex also leads to the disruption of the AF-1 site, resulting in a full antagonistic response (Pike *et al.,* 2001; Kong *et al.,* 2003).

Transcription of specific estrogen responsive genes occurs by one of two processes. In the first process, the ligand bound ER binds directly to specific DNA sequences within the promoter regions of the target genes called estrogen response elements (EREs). In the second process, the ligand bound ER interacts with other transcription factors within the cell, therefore stabilizing the binding of these transcription factors to different target genes not containing EREs. Both processes also require the binding of the ligand bound ER to coactivators and other elements of the transcriptional machinery, to activate the transcription of target genes (Dutertre and Smith, 2000).

In addition to the different SERM-ER complexes not being able to recruit the same coregulators and/or transcriptional factors that E_2 does, the change in receptor conformation induced by SERMs may also prevent the ER from binding to certain EREs (Dutertre and Smith, 2000). Therefore where the E_2 bound ER may activate the transcription of a gene with a certain ERE, the SERM bound ER may not recognize the same ERE and therefore the gene may not be transcribed, or may even be suppressed, and the effect of the SERM is therefore antagonistic. However, this may not be true of all estrogen target genes, as that particular SERM-ER complex may still be able to activate other estrogen responsive genes, containing different EREs that are recognized by both the SERM-ER complex and the E_2-ER complex. The same can be said for genes that are inhibited by E_2, which contain EREs that may not be recognized by the SERM-ER complex, and are therefore activated instead. This accounts for the mixed agonistic-antagonistic effects of many SERMs within a single cell or tissue type.

It has been shown that SERMs also have different effects on genes in different tissues and cells, where they may be antagonistic in one tissue or cell type, but agonistic in another tissue or cell type (Clark and Markaverich, 1982; Dutertre and Smith, 2000). These observed effects may be due to differences in expression of the coregulators in different cell types as well as a difference in the ratio of expression of the two receptor subtypes ERα and ERβ, which have similar LBDs and DNA binding domains (DBD's), but have different transactivational properties, gene expression and physiological effects (Ogawa *et al.,* 1998; Pike *et al.,* 1999). It has also been shown that the AF-1 transactivational domain is absent in ERβ and hence some partial agonists for ERα, which interfere only with the AF-2 domain, will act as pure antagonists for ERβ (Pike *et al.,* 1999). Certain SERMs only interfere with one of the AF sites (Hall *et al.,* 2001). Therefore those ligands, which only interfere with the AF-1 domain will only disrupt or prevent the association of the ER with a specific set of coactivators. If these coactivators are only found in one tissue or cell type, then those ligands will only act as an antagonist in that tissue or cell type. In the other tissues, which don't express these specific coactivators, the ligands will act as partial agonists (Pike *et al.,* 1999; 2001; Hall *et al.,* 2001).

Although the recent advances in receptor chemistry explain to some degree the action of SERMs, the specific action of CC with regard to the E_2 signaling pathway at the molecular level is still undetermined.

1.1.2 Clomiphene Citrate's Effects on the Uterus and Implantation

In addition to CC's effect on the pituitary and ovary to induce ovulation, CC has been shown to have a direct effect on the uterus. It has been shown to induce changes in uterine histology and ultrastructure (Poteat and Bo, 1977; Poteat, 1981; Dallenbach-Hellweg, 1988; Hosie *et al.,* 1991; Hosie and Murphy, 1992; 1995), as well as increase the expression of short chain oligosaccharides on the apical surface of the uterine luminal epithelium in ovariectomized rats, which were treated with hormones to induce receptivity to implantation (Hosie *et al.,* 1999; 2000; 2003). When CC was given to pregnant rats on days 2-4 of pregnancy and then sacrificed on day 5, which corresponds to the day of implantation, the nuclei of the uterine luminal epithelium were shown to be centrally placed, whereas the untreated 5 day pregnant rats had basally situated nuclei. This change in nuclear positioning has been associated with an accumulation of lipid below the nuclei (Poteat, 1981). In addition when CC was given to ovariectomized rats for 3 days, the glycogen content of the luminal epithelial cells of the uterus also increased compared to the uteri of untreated ovariectomized rats (Poteat and Bo, 1977). Both studies indicated that CC can interfere with the biochemical balance of the uterine luminal epithelium.

Clomiphene has been shown to decrease the endometrial thickness of patients treated with CC (Eden *et al.,* 1989; Rogers *et al.,* 1991). This decrease in the thickness of the endometrium has been shown to affect pregnancy outcome in women, as those patients who had an endometrial thickness of 9-10mm or more, following CC treatment, had better pregnancy success rates, than those who had an endometrial thickness of 6-8mm or less, following treatment (Dickey and Holtkamp, 1996). Clomiphene has also been shown to cause a desynchronization of the secretory pattern and a significant delay in the maturation of the endometrium, as well as a high incidence of out of phase endometria during the mid-secretory (mid-luteal) phase of the menstrual cycle. This has been shown in both anovulatory infertile and normal fertile women, treated with CC, when compared to untreated

women (Birkenfeld *et al.,* 1986; Dallenbach-Hellweg, 1988; Massai *et al.,* 1993; González *et al.,* 2001). In addition the mid-secretory phase of the menstrual cycle is known to corresponds to the phase of maximal endometrial receptivity to implantation in humans (Knobil and Neill, 1994). As a result of these studies, it has been suggested, that CC interferes with implantation, thus giving rise to the observed low pregnancy rates obtained in women treated with CC.

In animal studies, CC has been shown to decrease the implantation rates, in rabbits, from 62% to 18%, when administered before implantation (Birkenfeld *et al.,* 1985). Clomiphene has also been shown to decrease the number of implantation sites and full term pregnancies of donor embryos, which were transferred into prepubertal mice, given CC and a hormonal regime that induces implantation, when compared to vehicle, P_4 and E_2 treated mice (Nelson *et al.,* 1990). Clomiphene administered just before or at the time of implantation, also decreased the number of implantation sites in pregnant and pseudopregnant rats, when compared to untreated rats (Prasad and Kalra, 1967; Poteat, 1981; Cummings *et al.,* 1991; Barkai *et al.,* 1992). Furthermore it was shown that the adverse effect of CC on implantation, was most likely due to an adverse effect on the uterus, as it was shown to not have any cytotoxic effects on the blastocyst, nor did it have any adverse effects on the hypothalamic-pituitary-ovarian axis (Prasad and Kalra, 1967; Nelson *et al.,* 1990; Dickey and Holtkamp, 1996).

Although much research has been done on CC in clinical applications, light microscopic and a few electron microscopic and biochemical studies, little has been done on CC's effects on the uterus at the gene expression level. One study, showed that CC upregulates the α_1, α_V and β_3-integrins in the uterine stroma, and downregulates the β_3-integrins in the epithelium of normal ovulatory fertile women, during the mid-secretory phase of the menstrual cycle. The altered expression of these integrins by CC was therefore suggested to either prevent the attachment of blastocysts to the epithelium or subsequent invasion into the underlying stroma, or else prevent implantation through the non-synchronous development of the epithelium and stroma (González *et al.,* 2001). Although, this supports CC's adverse affect on endometrial receptivity to implantation, further work is still required to elucidate how this occurs at the gene expression level.

1.2 ESTROGEN, ESTROGEN RECEPTOR AND HSP90

Since CC is active in the uterus and binds to ERs present there (Clark and Markaverich, 1982), its effects may lie within the estrogen signaling pathway. It is therefore important to look at the signaling pathway activated by E_2, with particular focus on the ER and a chaperone protein, which is bound to the receptor in the absence of ligand: 90kDa heat shock protein (Hsp90) (Pratt and Toft, 1997).

1.2.1 Estrogen Signaling Pathway

Estradiol 17β enters the cell by passive diffusion and binds to ERs found within the nuclei of target cells (Grandien *et al.,* 1997). Both ERα and ERβ belong to the steroid/nuclear receptor superfamily that function as signal transcription factors and modulate the expression of specific target genes (Beato *et al.,* 1995; Grandien *et al.,* 1997; Klinge, 2001). ERα is the most prominent ER in the rat uterus, with very little expression of ERβ (Hiroi *et al.,* 1999; Wang *et al.,* 1999; 2000).

In the absence of E_2, ERα forms a heterocomplex with two Hsp90 molecules and one immunophilin, p59, molecule (Segnitz and Gehring, 1995). Hsp90 has been shown to bind to ERα's LBD as well as to a small region within its DBD (Chambraud *et al.,* 1990; Beato *et al.,* 1995; Pratt and Toft, 1997). Upon ligand binding, the ER undergoes a conformational change that transforms the receptor into a DNA binding form (Clark and Mani, 1994; Beato *et al.,* 1995). This transformation is accompanied by the dissociation of Hsp90 and homodimerization with another ligand bound ER (Pratt and Toft, 1997). Although there is still much debate as to the function of Hsp90, it has been suggested that it keeps the receptor in an inactive state, as well as in keeping the receptor in a high affinity ligand binding conformation, by facilitating the folding of the LBD (Grandien *et al.,* 1997; Pratt and Toft, 1997; Fliss *et al*., 2000). Triphenylethylenes, such as tamoxifen, and pure antiestrogens have also been shown to dissociate Hsp90 from the ER heterocomplex upon binding to the ER (Pratt and Toft, 1997; Devin-Leclerc *et al.,* 1998).

Following dimerization, the ligand bound ER dimer binds to EREs within the promoter regions of the E_2 target genes (Beato *et al.,* 1995; Pratt and Toft, 1997;

Klinge, 2001). The ER also binds to coactivators (or corepressors), that act as a bridge between the ER and the transcription initiation complex (Beato *et al.,* 1995; Grandien *et al.,* 1997; Klinge, 2001). The ligand-ER complex may also bind indirectly to target genes by binding to other transcription factors within the cell, which bind to different estrogen regulated genes and to different coactivators (or corepressors) (Klinge, 2001). Ultimately transcription (or suppression) of specific estrogen regulated genes is induced. The messenger RNAs (mRNAs) are then translated into proteins, which perform specific cell functions (Beato *et al.,* 1995; Grandien *et al.,* 1997).

1.2.2 Implantation and the Role of 17β Estradiol in Implantation

Implantation is a process that occurs 4-6 days after fertilization in both humans and rodents (Psychoyos, 1973). In humans this also corresponds to a period within the mid-secretory phase, between days 19-21, of the menstrual cycle (Finn and Porter, 1975). In rats, the blastocysts reach the uterus by the morning of day 5 of pregnancy. They become progressively surrounded by the luminal epithelium, thus bringing them into close contact with the apical surface of the uterine luminal epithelium. The blastocysts then attach to and penetrate the epithelium, around noon of day 5 (day 5½), and then invade into the underlying stroma. This always occurs on the antimesometrial side of the uterus in rodents (Enders and Schlafke, 1967; Psychoyos, 1973; Carson *et al.,* 2002).

During the first 5 days of pregnancy, the uterus undergoes several changes, under the influence of E_2 and P_4, which bind to their intracellular receptors and induce the transcription of genes that transform the uterus from a non-receptive state to a receptive state, termed the window of implantation (Psychoyos, 1973). This window only lasts for a few hours, such that by noon of day 6 of pregnancy, the uterine environment becomes hostile to non-implanted blastocysts and they are expelled through the vagina (Psychoyos, 1973; 1986). The changes that occur on the apical surface of the luminal epithelium, which is the site of first contact between the blastocyst and uterus, during this short period of receptivity, have been called the “plasma membrane transformation”, and this has been shown to occur in many species including rodents and humans (Murphy, 1995).

The changes encompassed in the plasma membrane transformation include: a flattening of the long microvilli that are present under the influence of E_2, prior to the window of receptivity; the presence of large bulbous cytoplasmic projections called pinopods (uterodomes in humans), particularly on the antimesometrial surface in rats and mice; and a reduction in the apical glycocalyx and surface negativity of the luminal epithelium (Enders and Schlafke, 1967; Psychoyos, 1973; Hosie and Murphy, 1989; Murphy, 1995; Adams *et al.,* 2002; Carson *et al.,* 2002).

Other changes that have been shown to occur in the uterus around the time of implantation include a disappearance of lipid from the luminal epithelial cells by noon of day 5 (day 5½), when implantation occurs. This lipid accumulates in the luminal epithelial cells prior to implantation and pushes the nuclei into a more apical position. However by day 5½ the nuclei are basally situated (Psychoyos, 1973; Poteat, 1981). The change in nuclear position has also been shown to occur around day 5 of the normal secretory phase of the menstrual cycle in humans (Dallenbach-Hellweg, 1988).

All these changes, including the uterine closure in rats and mice mentioned above, are under hormonal control, and can be induced in ovariectomized rats or mice with P_4 and/or E_2 treatment (Nilsson, 1967; Ljungkvist, 1971b; 1971c; 1972; Psychoyos, 1973; Poteat and Bo, 1977; Murphy and Rogers, 1981; Martel *et al.,* 1991; Knobil and Neill, 1994). In addition implantation of donor embryos can also be induced in ovariectomized rat and mouse uteri, with a specific hormone regime of P_4 treatment (priming) for at least 48 hours, followed by a single injection of a small dose of E_2 (Psychoyos, 1973).

Estradiol 17β is of particular importance in implantation, since the blastocysts never implant if ovariectomized rats are just primed with P_4. However, if in addition to the P_4 treatment, E_2 is given, and if the donor blastocysts are transferred within 24 hours of the E_2 treatment, implantation occurs (Psychoyos, 1973). Following this 24 hours, the uterus begins to revert back to a non-receptive state (Psychoyos, 1973). This hormonal induction of implantation has led several investigators to suggest that it is the condition of the uterus that determines the success or failure

of implantation, rather than factors contributed by the blastocyst (Yoshinaga and Adams, 1966a; Psychoyos, 1973; Paulson *et al.,* 1990).

Further support for E_2's role in initiating implantation is that if pregnant rats or mice are ovariectomized immediately before implantation, then implantation is blocked, but if the rodents are then given an exogenous source of E_2, blastocyst attachment is triggered (Yoshinaga and Adams, 1966a; 1966b; Gidley-Baird, 1981).

The role of E_2 in initiating implantation has also been shown in natural pregnancy. During the normal estrus cycle of rodents and during the normal menstrual cycle of humans, E_2 concentrations and secretion are generally high during proestrus in rodents, or the follicular (proliferative) phase in humans. However during metestrus in rats and mice, or during the luteal (secretory) phase in humans, E_2 concentration and secretion are generally low. During pregnancy in both species, E_2 levels are also low, except for a significant rise in E_2 secretion in the afternoon of day 4 of pregnancy, 24 hours before implantation (Yoshinaga *et al.,* 1969; Finn and Porter, 1975). This "E_2 surge" corresponds to the E_2 required to induce blastocyst attachment and receptivity in the ovariectomized P_4 primed rats discussed above.

1.2.3 Estradiol 17β Associated Uterine Changes for Implantation

Estradiol 17β has also been shown to stimulate many changes in the uterus, which have been suggested to be important in preparing a receptive uterine environment and therefore are associated with implantation. It stimulates hypertrophy and proliferation of the uterine epithelium and stroma, therefore resulting in an increase in the endometrial thickness and uterine weight (Poteat and Bo, 1977; Bowman *et al.,* 1981; Eden *et al.,* 1989; Brown *et al.,* 1991; Hosie and Murphy, 1992; Knobil and Neill, 1994). Estradiol 17β also increases the expression of short oligosaccharide chains that have been shown to increase during the implantation period in rats (Dutt *et al.,* 1988; Murphy and Turner, 1991; Hosie *et al.,* 1999). It has also been shown to increase the vascular permeability, of the endometrial capillaries, and blood flow to the uterus, resulting in stromal edema, as observed during the mid-secretory phase of the menstrual cycle and during implantation in both rodents and humans (Psychoyos, 1973; 1986; Curtis *et al.,* 1999).

The increase in vascular permeability that occurs during implantation has been said to be one of the earliest known responses of a receptive endometrium to implantation, such that by injecting a macromolecular vital dye, such as Pontamine Blue or Evans Blue, the sites of implantation can be marked as discrete blue bands along the uterine horns in rodents (Psychoyos, 1973). In mice, the increase in vascular permeability in response to E_2 treatment has been shown to be mediated through an increase in vascular endothelial growth factor (VEGF), which is known to be a potent inducer of increased microvascular permeability and endothelial cell mitosis (Rockwell *et al.,* 2002). It was also shown that no implantation occurred in pregnant mice when they were treated with anti-VEGF antibodies (Rockwell *et al.,* 2002). Furthermore the E_2 induced increase in vascular permeability has also been shown to be due to an increase in histamine and prostaglandin release in the uterus at the time of implantation (Psychoyos, 1986; Knobil and Neill, 1994; Curtis *et al.,* 1999).

In terms of the plasma membrane transformation, when E_2 is given to ovariectomized P_4 primed rats, it results in the loss of microvilli on the apical surface of the uterine luminal epithelium, which is typical of the uterine surface at implantation. However it has been shown that when E_2 is given to ovariectomized rats on its own, it results in an increase in the number and length of the microvilli (Nilsson, 1967; Ljungkvist, 1971c; 1972; Psychoyos, 1973; Hosie and Murphy, 1992; Murphy, 1995). E_2 has also been shown to remove the lipid that accumulates in uterine luminal epithelial cells during P_4 priming, thus changing the nuclear position in these cells from a central to a basal location (Psychoyos, 1973; Poteat and Bo, 1977; Knobil and Neill, 1994).

At the molecular level, E_2 has been shown to increase the expression of $\alpha_4\beta_1$ integrins in the uteri of ovariectomized P_4 primed mice, compared to ovariectomized mice primed only with P_4. These specific integrins have been linked to implantation, as no implantation occurs when pregnant mice are injected with antibodies against $\alpha_4\beta_1$ integrins (Basak *et al.,* 2002). This further supports the importance of E_2 in initiating blastocyst implantation.

It is also important to note that many of these changes brought about by E_2 in the uterus, have also been shown to be adversely affected by CC treatment and have

been suggested to be due to CC's antiestrogenic nature (Poteat and Bo, 1977; Poteat, 1981; Birkenfeld *et al.,* 1986; Dallenbach-Hellweg, 1988; Eden *et al.,* 1989; Hosie *et al.,* 1991; Rogers *et al.,* 1991; Hosie and Murphy, 1992; 1995; Massai *et al.,* 1993; Dickey and Holtkamp, 1996; Hosie *et al.,* 1999; 2000; 2003; González *et al.,* 2001).

1.2.4 Estrogen Receptor Alpha

Since ERα is the most prominent ER in the rat uterus (Hiroi *et al.,* 1999; Wang *et al.,* 1999; 2000), and has been shown to be present in mice uteri during the peri-implantation period by immunocytochemistry (ICC) and *in situ* hybridization (Tan *et al.,* 1999), it is therefore the most likely receptor subtype to mediate the effects of E_2 in inducing endometrial receptivity for implantation. It has also been shown that no implantation occurs, when donor embryos are transferred into the uterine lumen of ovariectomized ERα knockout mice, despite being treated with P_4 and E_2 to induce receptivity for implantation (Hewitt *et al.,* 2002). Stromal ERα has also been shown to mediate E_2 induced epithelial proliferation, which is important for preparing the uterus for implantation, and indicates the importance of epithelial-stromal interactions in the uterus (Cooke *et al.,* 1997).

The expression of ERα has been shown to be regulated by E_2. However regulation appears to be tissue specific. In the rat, E_2 decreases ER mRNA levels in the uterus, but increases ER mRNA in the liver and pituitary (Martin *et al.,* 1994). Several other studies have also shown a decrease in ERα mRNA and/or protein in the uterus, in response to E_2, possibly as a mechanism to control the physiological response of E_2 in estrogen target tissues (Horigome *et al.,* 1988; Rosser *et al.,* 1993; Zhou *et al.,* 1993; Nawaz *et al.,* 1999; Nephew *et al.,* 2000; Carley *et al.,* 2003). In contrast, others have shown that E_2 upregulates ERs in the rat uterus (Wang *et al.,* 1999), with ERα mRNA levels being shown to be highest during proestrus, when E_2 levels are highest, and lowest during metestrus, when E_2 levels are low (Wang *et al.,* 2000).

Triphenylethylenes, including CC, are able to bind to ERs (Clark and Markaverich, 1982; Jordan, 1984), and are therefore able to influence the expression of E_2 target genes. Since the concentration of ERα in the cell controls, to a large degree, the extent and duration of estrogen signaling, and since E_2 plays such an important role

in implantation, it would be of interest to test the effect of CC on the expression of ERα in the uterus. This may elucidate whether the changes in the uterus, observed as a result of CC treatment, arise from an effect at the level of the receptor in the estrogen signaling pathway. The effect of CC on ERα gene expression at implantation may also aid in explaining the poor implantation and pregnancy rates seen with CC treatment (Navot and Laufer, 1989; Cohen, 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000).

1.2.5 90kDa Heat Shock Protein

The effect of CC in the uterus may also be seen at the level of interaction between the ER and Hsp90. Heat shock proteins (Hsps), such as Hsp90, are a group of chaperone proteins that stabilize protein structure, renature unfolded proteins or target proteins for degradation (Lindquist, 1986).

Hsp90 is a highly abundant protein predominantly found in the cytoplasm of cells (Pratt and Toft, 1997). As mentioned previously, two Hsp90 molecules bind to a single ER, forming a heterocomplex in the absence of ligand (Segnitz and Gehring, 1995; Pratt and Toft, 1997). Many investigators have suggested that Hsp90 functions to stabilize steroid receptors, including the ER, in an inactive state and therefore prevents the receptor from binding to the DNA (Chambraud *et al.,* 1990; Clark and Mani, 1994). In support of this, earlier studies have shown that ERs and other steroid receptors are able to transform from a non-DNA binding form (9S complex) to a DNA-binding form (4S complex), and that this transformation occurs when Hsp90 dissociates from the receptor. This has led to the suggestion that Hsp90 prevents the ER from inducing the transcription of E_2 target genes (Pratt and Toft, 1997).

However, it has also been shown that except for a small C-terminal region of the DBD of ER, Hsp90 mostly binds to the LBD of the receptor (Chambraud *et al.,* 1990; Pratt and Toft, 1997). It is therefore not known how Hsp90 would prevent the ER from binding to the DNA. However, since the region required for dimerization is present in the LBD of ER, it has been suggested that Hsp90 may prevent dimerization, and that this, may somehow prevent the ER from binding to DNA (Pratt and Toft, 1997). In support of this idea, it has been shown that by

increasing the concentration of Hsp90 in cells, the amount of ER-ERE complexes decreased (Sabbah *et al.,* 1996). However, in the same study the exposure of the ER-Hsp90 complexes to vitellogenin A2 EREs (consensus ERE), resulted in the dissociation of Hsp90 from the ER-Hsp90 complex and the specific binding of the ERs to the EREs (Sabbah *et al.,* 1996). Furthermore others have shown that purified ERs (without Hsp90) from calf uterus have a very low binding capacity for the vitellogenin A2 ERE, and that the binding capacity of ERs to this ERE was strongly stimulated when purified ER was reconstituted with Hsp90. This suggested that Hsp90 stimulates the binding of ERs to EREs (Inano *et al*., 1994), in contrast to the studies by Sabbah *et al*., (1996). Therefore there appears to some debate as to whether Hsp90 inactivates ERs or not.

In experiments where the steroid receptor-Hsp90 heterocomplex formation is prevented, with the Hsp90 specific inhibitor geldanamycin, or where Hsp90 was dissociated from the receptor, reduced levels of hormone binding by the steroid receptor was observed (Pratt and Toft, 1997; Segnitz and Gehring, 1997; Fliss *et al.,* 2000). This has led to the suggestion that Hsp90 functions to maintain the ER in a high affinity hormone binding conformation. In addition the steroid binding capacity of the receptors was shown to be restored upon withdrawal of geldanamycin (Segnitz and Gehring, 1997).

Hsp90 has also been suggested to play a protective role for steroid receptors, due to studies where rapid degradation of ERs and glucocorticoid receptors (GRs), by the ubiquitin proteasome pathway, occurred when Hsp90 functioning was inhibited with radicicol or geldanamycin (Pratt and Toft, 1997; Lee *et al.,* 2002). Degradation of ERs by the ubiquitin proteasome pathway has also been shown to occur in MCF7 (breast carcinoma) and lactotrope (pituitary) cell lines in response to E_2 (Alarid *et al.,* 1999; Nawaz *et al.,* 1999; Lonard *et al.,* 2000). As mentioned already, E_2 is also known to dissociate Hsp90 from the receptor (Pratt and Toft, 1997). Therefore when E_2 binds to the ER it must somehow induce a conformation within the ER which no longer allows the Hsp90 to be bound to the ER. As Hsp90 dissociates from the receptor it probably uncovers ubiquitin binding sites, thus making the ER susceptible to degradation by the ubiquitin proteasome pathway.

The above studies, particularly those with geldanamycin and radicicol, which support a role for Hsp90 in maintaining ERs in a high affinity ligand binding conformation, also support a role for Hsp90 in activating the ligand induced transcriptional activity of ERs. Further evidence for such a role also includes observations that the transcriptional activity of ERs on ERE containing genes, in the presence of E_2, decreases when Hsp90 is inhibited with radicicol, compared to untreated cells (Lee *et al.,* 2002). Steroid receptors, including ER, have also been shown to be less efficient in activating transcription of steroid responsive genes, in the absence of, or with reduced levels, of Hsp90, or when cells are treated with geldanamycin (Beato *et al.,* 1995; Grandien *et al.,* 1997; Pratt and Toft, 1997; Segnitz and Gehring, 1997). It is therefore feasible that the level of Hsp90 expression may influence the transcriptional activity of the ER in the uterus, thus altering the expression of estrogen regulated genes, which may be involved in preparing the uterus for implantation.

Hsp90 expression has been shown to be upregulated by E_2 in rat (Olazabal *et al.,* 1992), mouse (Ramachandran *et al.,* 1988; Papaconstantinou *et al.,* 2001) and sheep uteri (Wu *et al.,* 1996). It was also suggested that this increase in Hsp90 may serve to stimulate ER function in the uterus (Wu *et al.,* 1996). The Hsp90 gene has also been shown to contain a single but imperfect ERE further supporting the estrogenic regulation of this gene, through the ER (Koshiyama *et al.,* 1995). Given that SERMs also interact with the ER, it is possible that Hsp90 expression may also be regulated by SERMs such as CC and, therefore it would be of interest to test the effect of CC on Hsp90 expression in the rat uterus. This would also establish whether the effect of CC on the uterus, is at the level of Hsp90. This may also establish whether CC interferes with the ability of ERs to efficiently activate the transcription of estrogen regulated genes, involved in initiating implantation.

1.3 HOXA10 AND ITS ROLE IN IMPLANTATION

In order to investigate the assumption that CC affects blastocyst implantation in the uterus, and show such an effect at the gene expression level, it would be of interest to test the effects of CC on the expression of genes in the uterus, known to be involved in implantation. One particular gene that has been shown to be regulated by both E_2 and P_4 and is known to be involved in implantation is Hoxa10 (Taylor, 2000).

1.3.1 Homeobox Genes

Homeobox (Hox) genes are highly evolutionary conserved transcription factors that are essential for regulating differentiation and morphogenesis in the embryonic development of invertebrates and vertebrates (McGinnis and Krumlauf, 1992; Krumlauf, 1994). Hox genes have been shown to play essential roles in assigning tissue and cell identity to body segments along the anterior-posterior axis in the embryo, by binding to and activating or suppressing genes that determine the identity of that segment (Krumlauf, 1994). Hox genes are expressed sequentially along the anterior-posterior body axis of the embryo, in the same order as they are arranged along the chromosome (McGinnis and Krumlauf, 1992). Mutations in only one of these genes in *Drosophila* causes major transformations, such that one body segment will develop with characteristics of another. However in vertebrates, such mutations only result in subtle transformations of the region where the mutated genes are expressed, into a more anterior-like structure (Taylor, 2000).

1.3.2 The Hoxa Cluster of Homeobox Genes

The Hoxa cluster of homeobox genes in vertebrates is related to the *Drosophila* AbdominalB genes, which are expressed in the posterior of the fly (Taylor, 2000). In mammalian female embryos Hoxa9, Hoxa10, Hoxa11 and Hoxa13, are expressed consecutively in an anterior-posterior direction along the paramesonephric duct, which develops into the female reproductive tract (Taylor *et al.,* 1997). Hoxa9 is expressed in the most anterior part of the paramesonephric duct, which develops into the oviduct (Fallopian tube) in the adult. Hoxa10 is expressed in the region of the paramesonephric duct, which develops into the uterus. Hoxa11 is expressed in

the region which gives rise the lower part of the uterus and the cervix and where Hoxa13 is expressed, becomes the upper vagina (Taylor *et al.,* 1997).

Generally Hox expression is restricted to during embryonic development; however it was found that Hoxa9-13 expression persisted in adult reproductive tissues, in the same pattern as observed in the embryo, of both mice and humans. Hoxa9 is therefore expressed in the oviduct, Hoxa10 and Hoxa11 are expressed in the uterus, Hoxa11 is also expressed in the cervix, and Hoxa13 is expressed in the vagina, in both adult mouse and human tissue (Benson *et al.,* 1996; Taylor *et al.,* 1997). Since the endometrium is dynamic and undergoes continuous cyclic changes in cell proliferation, growth, differentiation and apoptosis, which are events that commonly occur in embryonic development, it was suggested that the persistent Hox expression enabled the reproductive tract, particularly the endometrium to remain in a state of developmental plasticity. In other words, it is postulated that the persistent expression of Hox genes in the adult enables the endometrium to undergo changes during the menstrual cycle and in pregnancy (Taylor *et al.,* 1997; Taylor, 2000).

1.3.3 Hoxa10 and Implantation

Female mice carrying a targeted disruption in the Hoxa10 gene (Hoxa10 -/-) were found to be fully viable but infertile. These Hoxa10 -/- mutant mice ovulated normally and produced normal embryos, which when transferred into wildtype surrogate mice, developed normally. However, the mutant mice were unable to support the survival of embryos around the time of implantation and wild type embryos were unable to implant when transferred into the uteri of Hoxa10 -/- mutant mice, suggesting a uterine cause for the apparent infertility (Satokata *et al.,* 1995; Benson *et al.,* 1996).

The uterine structure in the Hoxa10 -/- mutant mice showed that the proximal 25% of the uterus was transformed into an oviduct-like morphology, with both histology and molecular character being affected, however the majority of the uterus appeared morphologically and histologically normal (Satokata *et al.,* 1995; Benson *et al.,* 1996). This finding suggested that there is a homeotic transformation of the proximal 25% of the uterus to a more anterior-like identity in the mutant mice. However

implantation and early post-implantation survival of embryos failed to occur in these mutant mice, even when wildtype embryos were transferred into the distal (untransformed) region of the Hoxa10 -/- uterus (Satokata *et al.,* 1995; Benson *et al.,* 1996). This finding indicated that the infertility of these knockout mice is probably not due to an embryonic defect in the development of their uteri, but rather due to an absent functional requirement of Hoxa10 expression, in the adult, during pregnancy.

In delayed implantation experiments mated mice or rats are ovariectomized before implantation, and are given daily P_4 injections, thus eliminating the estrogenic stimulus on the uterus, but maintaining the P_4 effects. In these experiments, the blastocysts do not implant, but lie free in the uterus and are viable but dormant. Delayed implantation can be activated upon a single injection of E_2, in which the blastocysts will then implant (Yoshinaga and Adams, 1966a; 1966b; Psychoyos, 1973). When implantation was experimentally delayed in the Hoxa10 -/- mutant mice, the mice were able to support the survival of the blastocysts, equal to that observed in wildtype mice. Therefore, this suggested that the function of Hoxa10 was intrinsic to implantation (Benson *et al.,* 1996). By blocking maternal Hoxa10 expression in pregnant wildtype mice, with DNA/liposome complexes containing Hoxa10 antisense oligonucleotides, blastocyst implantation was also inhibited (Bagot *et al.,* 2000; Taylor, 2000). In the same study, when Hoxa10 was overexpressed in the pregnant wildtype mice, the resulting litter sizes were increased, compared to the untreated mice (Bagot *et al.,* 2000). These studies further support a role for Hoxa10, in the preparation of the endometrium for implantation, in the adult.

Hoxa10 has been shown to be expressed in the glandular and stromal cells of the endometrium throughout the menstrual cycle in humans (Taylor *et al.,* 1998). Both Hoxa10 mRNA and protein are low during the proliferative phase but increase markedly in the stromal and epithelial cells during the mid-secretory phase of the menstrual cycle, which corresponds to the time when the uterus transforms to a state of receptivity for implantation, with the expression remaining high throughout the rest of the secretory phase (Taylor *et al.,* 1998; Gui *et al.,* 1999). Expression of Hoxa10 has also been determined during early pregnancy in the mouse. On days

0.5-1.5 of pregnancy, Hoxa10 mRNA was detected in the luminal and glandular epithelium of wildtype mice uteri by *in situ* hybridization. A shift in Hoxa10 localization was shown to occur by day 2.5, such that by day 3.5 of pregnancy, which corresponds to the day before implantation in the mouse, Hoxa10 mRNA was detected only in the stroma. Hoxa10 also continued to be strongly expressed, in the developing decidua, after implantation between days 4.5 and 7.5 of pregnancy (Satokata *et al.,* 1995).

Stromal cell proliferation in response to P_4, which is important in preparing the endometrium for implantation, has been shown to be impaired in Hoxa10 -/- mutant mice. However, epithelial cell proliferation in response to both P_4 and E_2 was not affected in the same mice (Lim *et al.,* 1999). The increase in vascular permeability that occurs in implantation and is said to be an indicator of a receptive endometrium for implantation (Psychoyos, 1973), was also shown to be reduced in Hoxa10 -/- mutant mice (Benson *et al.,* 1996). This was shown by the observation that when 4.5 day pregnant Hoxa10 -/- mutant mice were injected with Evans blue, to localize implantation sites, the blue dye reaction was only faintly visible, diffusely spread or even absent, compared to the wild-type mice, which had discrete, strongly positive blue bands at the sites of implantation (Benson *et al.,* 1996). On the other hand, overexpression of Hoxa10 in pregnant mice produces more pinopods on the uterine surface, at the time of maximal uterine receptivity to implantation, than in normal pregnant mice. By decreasing the expression of Hoxa10 with antisense oligonucleotides, the number of pinopods decreased in pregnant mice at the time of implantation, compared to controls (Bagot *et al.,* 2001). It was therefore suggested in this study that the expression of Hoxa10 may be a key factor in the expression of pinopods (Bagot *et al.,* 2001).

All these studies indicated that Hoxa10 may play a key role in preparing the endometrium for implantation. They also indicate that Hoxa10 is not only required for changes in the stroma, such as in stromal cell proliferation and vascular permeability, but also for the transformation of the epithelium, due to its effect on the presence of pinopods, during implantation (Benson *et al.,* 1996; Lim *et al.,* 1999; Bagot *et al.,* 2001). It has also been shown that women with reduced fertility due to

altered endometrial development, endometriosis, or type II defects in endometrial receptivity, have lower levels of Hoxa10 mRNA in the endometrial stroma during the mid-secretory phase of the menstrual cycle, compared to normal fertile controls (Taylor *et al.,* 1998; 1999; Gui *et al.,* 1999). Therefore it seems that the functional requirement of Hoxa10 in implantation is conserved in humans as well as in rodents and is possibly responsible for infertility in women as a result of a loss of uterine receptivity, during the implantation window.

1.3.4 Hoxa10 and Genes Involved in Implantation

Although the mechanism in which Hoxa10 achieves endometrial receptivity for implantation is unknown, a number of genes have been shown to be altered when Hoxa10 expression is altered. Two cyclin dependent kinase inhibitors (p57 and p15), known to cause cell cycle arrest, were more highly expressed in Hoxa10 -/- mutant mice, than in wildtype mice (Yao *et al.,* 2003). These genes have been suggested to account for the decrease in stromal cell proliferation observed in these mice (Lim *et al.,* 1999; Yao *et al.,* 2003).

Prostaglandins have been implicated in implantation, due to their vasoactive and mitogenic activities, and are synthesized via the rate limiting enzyme cyclooxygenase (COX) (Lim *et al.,* 1997; 1999). It has been shown that the stromal prostaglandin binding cell surface receptors (EP3 and EP4) mRNA expression is significantly reduced in day 4 (implantation day) pregnant Hoxa10 -/- mutant mice, compared to wild-type. The upregulation of these two genes, in response to P_4 treatment was also not observed in the Hoxa10 -/- mutant mice. Furthermore the aberrant expression of EP3 and EP4 mRNA in these mice was not due to a deficiency in other genes, such as ERs or progesterone receptors (PRs), indicating that Hoxa10 is somehow responsible for mediating the effects of P_4 on EP3 and EP4 expression in the uterine stroma (Lim *et al.,* 1999).
Cyclooxygenase-2 has also been shown to be essential for implantation and stromal COX-2 induction, at the time of blastocyst attachment, was also significantly reduced or absent in the Hoxa10 -/- mutant mice uteri, compared to the wildtype mice suggesting that Hoxa10 is upstream of COX-2 expression (Lim *et al.,* 1997; 1999).

In another study, β_3 integrin subunit mRNA levels were shown to be significantly decreased in endometrial biopsies from patients with endometriosis, which are known to have significantly lower Hoxa10 mRNA expression, in the mid-secretory phase of the menstrual cycle compared to fertile controls (Daftary *et al.,* 2002). β_3 integrin mRNA levels were also significantly increased in Ishikawa cells (endometrial adenocarcinoma cell line) when the cells were transfected with plasmid constructs that constitutively express Hoxa10, and β_3 integrin mRNA levels decreased when cells were treated with Hoxa10 antisense oligonucleotides, compared to control missense-construct treated cells. Hoxa10 is also suggested to directly regulate the β_3 integrin gene, as it contains 7 consensus Hoxa10 binding sites, to which the Hoxa10 protein was found to bind to 4 of these sites. A 41bp (base pair) region containing two of these sites was also found to drive reporter gene expression in response to Hoxa10 (Daftary *et al.,* 2002). Integrins are cell adhesion molecules that participate in cell-cell and cell-extracellular matrix interactions in all cell types (Lessey, 2003). They have also been suggested, particularly $\alpha_v\beta_3$, to play a role in attaching the blastocyst to the maternal uterus during implantation and are said to be markers of uterine receptivity (Lessey *et al.,* 1992; Daftary *et al.,* 2002; Lessey, 2003).

The expression of several T cell genes and the number of T cells in the uterine stroma were also shown to be increased in the Hoxa10-/- mutant mice compared to wildtype mice (Yao *et al.,* 2003). The expression of such genes and the presence of these cells would increase the cytolytic and inflammatory activities of the stroma. Since implantation involves the attachment of a foreign body (blastocyst) to the maternal tissue, it has been suggested that this increase in immune related factors would adversely affect implantation (Yao *et al.,* 2003). In support of this theory, it was observed in a study where microarray analysis was used to determine the difference in gene expression between non-implantation and implantation sites and between delayed and activated implantation uteri in the mouse, that many of the genes that were downregulated at the implantation sites and in the activated implantation uterus, were immune related genes (Reese *et al.,* 2001).

Several other genes observed by Reese *et al.,* (2001) to be differentially regulated in implantation and non-implantation sites, or between activated and delayed implantation uteri, were also found to be differentially regulated between Hoxa10 -/- mutant and wildtype mice uteri (Yao *et al.,* 2003). This suggests that the role of these genes in implantation is in some way mediated by Hoxa10 (Yao *et al.,* 2003). These genes included: follistatin, BiP, leptin receptor, transforming growth factor β (TGF-β); glutathione-S-transferase (GST) theta 1; aquaporin 1; inhibitor of DNA binding 1; thioether S-methyltransferase; insulin-like growth factor (IGF) binding protein 3 and Hoxa11. However the specific functions and expression pattern during the peri-implantation period of most of these genes has not yet been determined (Reese *et al.,* 2001; Yao *et al.,* 2003).

In conclusion this and other studies have suggested that Hoxa10 is responsible for mediating the effects of the ovarian hormones, particularly P_4, during implantation thus giving rise to a receptive endometrium for implantation in mammals including mice and humans (Lim *et al.,* 1999; Yao *et al.,* 2003).

1.3.5 Hormonal Control of Hoxa10

Hoxa10 has also been shown to be regulated by the ovarian hormones E_2 and P_4. Estradiol 17β upregulates the expression of Hoxa10 mRNA in epithelial and stromal cells isolated from human endometrial tissue as well as in the Ishikawa cell line by 2 fold (Taylor *et al.,* 1998; Gui *et al.,* 1999). In mice, however, E_2 was shown to inhibit the uterine stromal expression of Hoxa10 (Ma *et al.,* 1998).

In both human and mice uterine studies, P_4 also upregulated Hoxa10 mRNA expression and the increase observed was greater than that induced by E_2 alone (Ma *et al.,* 1998; Taylor *et al.,* 1998; Gui *et al.,* 1999), such that in the human endometrial stromal cells and in the Ishikawa cell line P_4 upregulated Hoxa10 expression by as much as 25 fold (Taylor *et al.,* 1998). However maximal expression of Hoxa10 mRNA occurred when human endometrial epithelial and stromal cells were treated with a combination of E_2 and P_4 (Taylor *et al.,* 1998; Gui *et al.,* 1999). However, the combined treatment of E_2 and P_4 resulted in a restricted pattern of Hoxa10 expression to the antimesometrial side in mice uteri, where blastocyst implantation in rodents

occurs (Ma *et al.,* 1998). As mentioned before implantation of donor embryos can be induced in ovariectomized rodents by treating them with P_4 for at least 48 hours, followed by a single dose of E_2, which is given in combination with P_4 (Psychoyos, 1973). The pattern of Hoxa10 gene expression, during the menstrual cycle, pregnancy and following ovarian steroid treatment in both mice and humans, further strengthens Hoxa10's role in implantation. These studies also suggest that Hoxa10 mediates the effects of E_2 and P_4 in the uterus particularly during implantation by activating endometrial downstream genes leading to receptivity for implantation (Lim *et al.,* 1999; Cermik *et al.,* 2001).

The Hoxa10 gene in humans has also been shown to contain an ERE, which is able to bind to both ERα and β and mediate E_2-responsive transcriptional activation in the Ishikawa cell line (Akbas *et al.,* 2004). In addition the induction of Hoxa10 expression by E_2 is not affected by the protein synthesis inhibitor cycloheximide in both mice and humans, indicating that Hoxa10 is a direct target of the ER, and further supports an estrogenic regulation of Hoxa10 (Ma *et al.,* 1998; Taylor *et al.,* 1998). In human endometrial cells the pure antiestrogen ICI 182,780 decreased Hoxa10 mRNA levels, in contrast to the increase observed with E_2 (Gui *et al.,* 1999). Since CC binds to ERs it is possible that CC may also have an effect on the expression of Hoxa10, which may begin to explain how CC affects implantation at the gene expression level, thus giving rise to poor pregnancy rates in women treated with CC.

1.4 AIM AND OBJECTIVES OF THE STUDY

In order to elucidate the action of CC at the gene level and its role in the observed low implantation and pregnancy rates, this study tested the effect of CC in the expression of three genes: ERα, Hsp90 and Hoxa10 in the rat uterus.

Specific aims of the study were:

1) To test and compare the effect of a single dose of CC or E_2 on the gene expression of ERα and Hsp90, which are involved in the E_2 signaling pathway, in the ovariectomized rat uterus, that is in the absence of ovarian hormones.
2) To test the effect of CC on ERα and Hsp90 expression at implantation by treating rats with CC prior to a hormone regime that can induce implantation in the rat uterus (pseudopregnancy).
3) To test and compare the effect of a single dose of CC or E_2 in the ovariectomized rat uterus on the expression of Hoxa10, which is known to be directly involved in preparing the uterus for implantation in both rodents and humans.
4) To test the effect of CC on the expression of Hoxa10 at implantation in the pseudopregnant rat uterus.