# ESTROGEN-MEDIATED MODULATION OF UROPATHOGENIC Dr+ *ESCHERICHIA COLI*-INDUCED UROPATHOGENESIS

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ESTROGEN-MEDIATED MODULATION OF

UROPATHOGENIC Dr+ *ESCHERICHIA COLI*-

INDUCED UROPATHOGENESIS

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Akt | A serine/Threonine protein kinase also known as Protein Kinase B (PKB) |
| CFU | Colony forming unit |
| Col4A1 | Type IV collagen subtype A1 |
| DAEC | Diffusely adherent *Escherichia coli* |
| DAF | Decay accelerating factor |
| DPN | Diarylproionitrile |
| E2 | 17-β-estradiol |
| *E. coli* | *Escherichia coli* |
| Dr+ *E. coli* | Dr adhesin bearing *E. coli* |
| ER | Estrogen receptor |
| ERα | Estrogen receptor alpha |
| ERβ | Estrogen receptor beta |
| ERα-/- | Estrogen receptor alpha gene knock-out mice |
| ERα+/+ | Estrogen receptor alpha gene intact mice |
| ERE | Estrogen Response Element |
| GPI | Glycosyl phosphotidylinositol |
| ICI | ICI 182, 780 |
| IL8 | Interleukin |

| | |
|---|---|
| mIMCD | Mouse inner medullary collecting duct cells |
| hIMCD | Human inner medullary collecting duct cells |
| LA | Luria Bertani agar |
| LPS | Lipopolysachharide |
| MPP | Methylpiperidin pyrazole |
| MIP2 | Macrophage Inflammatory Protein-2 |
| MAPK | Mitogen activated protein kinase |
| Non-OVX | Non-ovariectomized (ovary intact) |
| OVX | Ovariectomized |
| PAMP | Pathogen associated molecular patterns |
| PBS | Phosphate buffered Saline |
| pAkt | Phosphorylated Akt. |
| PI3K | Phosphoinositide3- Kinase |
| pPI3K | Phosphorylated PI3K. |
| PMSF | Phenylmethanesulfonyl fluoride |
| PPT | Propylpyrazoletriol |
| PPIA | Peptidylprolyl isomerase A |
| PRR | Pathogen recognition receptors |
| R, R-THC | R, R-tetrahydrochrysene |
| TLR | Toll like receptor |
| TNFα | Tumor necrosis factor alpha |
| UPEC | Uropathogenic *Escherichia coli* |
| UTI/UTIs | Urinary tract infection/infections |

# CHAPTER I

# INTRODUCTION

## 1.1 Background

Urinary tract infections (UTIs) are the most frequently acquired bacterial infections by humans (Drekonja & Johnson, 2008). Women are more prone to UTIs than men. About 40-50 % of women in all age groups are estimated to acquire UTIs during their lifetime (Brown *et al*, 2005, Foxman *et al*, 2003, Franco, 2005, Zhang & Foxman, 2003). Despite successful and effective antimicrobial treatment, UTIs remain a significant clinical problem accounting for more than 6 billion dollars annually in healthcare (Drekonja & Johnson, 2008, Foxman, 2003). Development of drug resistant bacterial strains has added to the complications and has increased the frequency of recurrent UTIs (Blango & Mulvey, 2010, Drekonja & Johnson, 2008, Mazzulli, 2002). Thus, a critical need exists for alternative therapeutic strategies to complement antibiotic treatment against UTIs.

Incidence of UTIs in females seems to vary with age and stages of menstrual cycle suggesting hormonal regulation of susceptibility to UTIs (Stamm & Raz, 1999, Valiquette, 2001). Post-menopausal women have high prevalence of UTIs and estrogen

deficiency is considered an important contributing factor (Altoparlak *et al*, 2004, Foxman *et al*, 2001, Franco, 2005, Hu *et al*, 2004, Pabich *et al*, 2003). Exogenous estrogen replacement therapy in postmenopausal women is effective in preventing UTIs and ameliorating symptoms associated with infection and inflammation (Devillard *et al*, 2004, Heinemann & Reid, 2005, Maloney, 2002, Rozenberg *et al*, 2004, Stern *et al*, 2004). Beneficial effects of estrogen-treatment in postmenopausal females may include restoration of the periurethral and vaginal microflora and reduction in the risk of vaginal atrophy; however molecular mechanisms associated with the therapeutic effects of estrogen against UTIs are poorly understood (Cardozo *et al*, 1998, Cardozo *et al*, 2001, Rozenberg *et al*, 2004).

Estrogen is considered to be an anti-oxidant and anti-inflammatory agent that acts via the estrogen receptor (ER) subtypes, ER alpha (ERα) and ER beta (ERβ), to mediate regulation of gene transcription (Nilsson *et al*, 2001, Nilsson & Gustafsson, 2002). Expression of ERα and ERβ varies with circulating estrogen levels in humans and in animals (Ben-Hur *et al*, 1995, Esqueda *et al*, 2007, Rogers *et al*, 2007). Furthermore, reduced ER subtype expression in rat and mouse urogenital tissues following ovariectomy is found to be restored upon β-estradiol (E2) replacement (Carley *et al*, 2003, Esqueda *et al*, 2007, Rogers *et al*, 2007). Moreover, in kidney ERα is the predominant subtype expressed and is reported to be the primary mediator of estrogen regulated gene expression in the renal cells (Jelinsky *et al*, 2003). However, the effects of estrogen/ERα action in the kidney during uropathogenic infections are not completely

understood. Thus, a complete understanding of the molecular mechanisms underlying the estrogen related etiology of UTIs pathogenesis is warranted.

Uropathogenic *Escherichia coli* (UPEC) are the primary etiological agents (80%) of acute and chronic UTIs, including cystitis and pyelonephritis (Czaja *et al*, 2007, Marrs *et al*, 2005, Ronald, 2002, Zhang & Foxman, 2003). Colonization of the urinary tract by virulent UPEC strains occurs via various adhesins, including type I fimbriae, P pili, and Dr adhesins (Hagberg *et al*, 1983b, Johnson & Russo, 2005, Svanborg & Godaly, 1997). Dr adhesion expressing *E. coli* (Dr+ *E. coli*) are associated with cystitis, recurrent UTIs, and pyelonephritis in humans (Goluszko *et al*, 1997, Nowicki *et al*, 2001, Servin, 2005). Dr+ *E. coli* colonize the uroepithelium via Dr adhesin specific tissue receptors, decay accelerating factor (DAF, also known as CD55) and type IV collagen (Nowicki *et al*, 2001, Selvarangan *et al*, 2000, Selvarangan *et al*, 2004). Dr adhesin of Dr+ *E. coli* has been found to be critical for the development of chronic pyelonephritis in an experimental UTI C3H/HeJ mouse model (Goluszko *et al*, 1997, Nowicki *et al*, 2001). Binding of Dr adhesin to DAF allows attachment and colonization of Dr+ *E. coli* to the uroepithelium; while attachment of Dr adhesins to type IV collagen allows persistence of Dr+ *E. coli* within the tissue (Nowicki *et al*, 2001, Selvarangan *et al*, 2000, Selvarangan *et al*, 2004). Following infection, engagement of Dr+ *E. coli* with DAF on the host uroepithelium induces recruitment of additional DAF around the bacteria, leading to internalization into the epithelial cells (Das *et al*, 2005, Goluszko *et al*, 1999, Guignot *et al*, 2001, Kansau *et al*, 2004, Plancon *et al*, 2003, Selvarangan *et al*, 2000). Internalization of UPEC including Dr+ *E. coli* by the uroepithelial cells has been found to

be associated with tyrosine phosphorylation of specific host proteins (Guignot *et al*, 2001, Palmer *et al*, 1997, Peiffer *et al*, 1998). Moreover, tyrosine kinase pathway is activated following engagement of DAF with its ligands (Abedi & Zachary, 1997, Burridge *et al*, 1988, Shenoy-Scaria *et al*, 1992, Stefanova *et al*, 1991, Zachary & Rozengurt, 1992). Expression of DAF and type IV collagen is reported to be hormonally regulated (Potier *et al*, 2001, Potier *et al*, 2002, Seliger *et al*, 2001, Silbiger *et al*, 1998, Song *et al*, 1996). Estrogen has also been shown to modulate tyrosine kinase activity (Gao & Yamaguchi, 2000, Li & Yu, 2003, Liu & Howard, 1991, Pascoe & Oursler, 2001, Yoneda *et al*, 1993). Nonetheless, modulation of DAF and type IV collagen expression, and regulation of tyrosine kinase activation by estrogen during Dr+ *E. coli* uropathogenesis have not yet been investigated.

Pathology during UTIs is caused by UPEC colonization and inflammation induced tissue damage (Brogden *et al*, 2005, Svanborg *et al*, 1999, Svanborg *et al*, 2001a, Zasloff, 2006, Zasloff, 2007). Renal pyelonephritis is caused by ascent of UPEC from the bladder to the kidney followed by inflammation induced damage to the pelvic and renal tubules (Svanborg *et al*, 2001a, Vandewalle, 2008). Animal and clinical studies have shown that the uroepithelial toll like receptors (TLRs) including TLR2, TLR4, TLR5, and TLR11, play a central role in UPEC recognition to initiate host innate inflammatory responses against invading pathogen, and these responses further lead to specific activation of adaptive immunity (Chowdhury *et al*, 2006, Hawn *et al*, 2009a, Hawn *et al*, 2009b, Svanborg *et al*, 2001b, Tseng *et al*, 2007, Vandewalle, 2008, Wullt *et al*, 2001). For instance, TLR4 binds to lipopolysacharide (LPS) of gram negative UPEC. Upon ligand

binding, TLR4 is activated to stimulate transcription and secretion of pro-inflammatory cytokines (TNFα, IL6, Il-1β) and chemokines (IL8/MIP2, RANTES, MCP1), as well as up-regulation of TLR2 activation (Chassin *et al*, 2006, Chowdhury *et al*, 2006, Samuelsson *et al*, 2004, Schroppel & He, 2006, Vandewalle, 2008). TLR4 signaling defects cause LPS unresponsiveness, thus increases susceptibility to gram negative bacterial infections and UTIs in animals and humans (Agnese *et al*, 2002, Hawn *et al*, 2009a, Karoly *et al*, 2007, Poltorak *et al*, 1998, Ragnarsdottir *et al*, 2007). Immunomodulatory roles of estrogen are well documented; however, to our knowledge the regulatory effects of estrogen on immune response associated with uropathogenic infection have not been investigated.

Estrogen appears to be an effective modulator of UPEC colonization in the female urinary tract; however the benefits of estrogen treatment in postmenopausal UTI susceptibility are poorly understood (Cardozo *et al*, 1998, Cardozo *et al*, 2001, Elloso *et al*, 2005, Rozenberg *et al*, 2004). **In this study, we aim to investigate the role of estrogen in host/pathogen interactions and inflammatory responses during Dr+ *E. coli* induced UTIs by utilizing an *in vivo* experimental UTI murine model and an *in vitro,* kidney cell culture model. The long term goals of this study are to understand the cellular and molecular regulatory mechanisms involved in estrogen mediated modulation of host susceptibility to Dr+ *E. coli* induced UTIs. This information will contribute toward the understanding of UTI pathogenesis, help in identification of novel biomarkers, and guide in the development of novel therapeutic strategies other than antibiotics for effective treatment of UTIs.**

## 1.2 Preliminary Studies

Our preliminary *in vivo* and *in vitro* studies indicate an increase in susceptibility to Dr+ *E. coli* infection under estrogen-deficient conditions and support the protective role of physiological doses of E2 against Dr+ *E. coli* infection. We have determined the impact of ovariectomy induced menopause on susceptibility towards Dr+ *E. coli* induced experimental UTI using the C3H/HeJ mouse model at 21 days post-infection. The ovariectomized (OVX) mice treated with a physiological dose of E2 and the estrogen sufficient-ovary intact (Non-OVX) mice show reduced bacterial colonization and infection related tissue inflammation in the kidneys, compared to the colonization in the kidneys of estrogen-deficient-vehicle-treated OVX mice (Figures 1 and 2). Pretreatment of Non-OVX with ER-antagonist ICI 182780 led to a significant increase in bacterial colonization of the kidneys, compared to that in the kidneys of vehicle-treated Non-OVX mice (Figure 3). These observations suggest an ER mediated protective role of E2 at a physiological dose against UTI in C3H/HeJ mice.

Quantitative real-time RT-PCR analysis revealed that ERα is more abundantly expressed than ERβ in the kidneys of C3H/HeJ mice and that ovariectomy decreased ERα mRNA levels in the kidneys of these mice, with no change in ERβ expression (Figure 4A). Notably, the vehicle-treated OVX mice showed increase in ERα mRNA levels in the kidney following infection, compared to the uninfected OVX mice but the infected Non-OVX mice showed decrease in ERα mRNA levels, compared to the uninfected Non-OVX mice (Figure 4A and 4B).

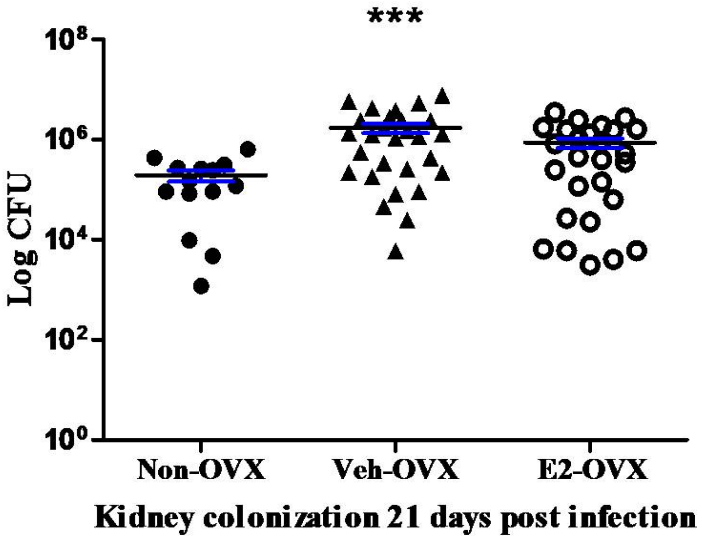


**Figure 1**

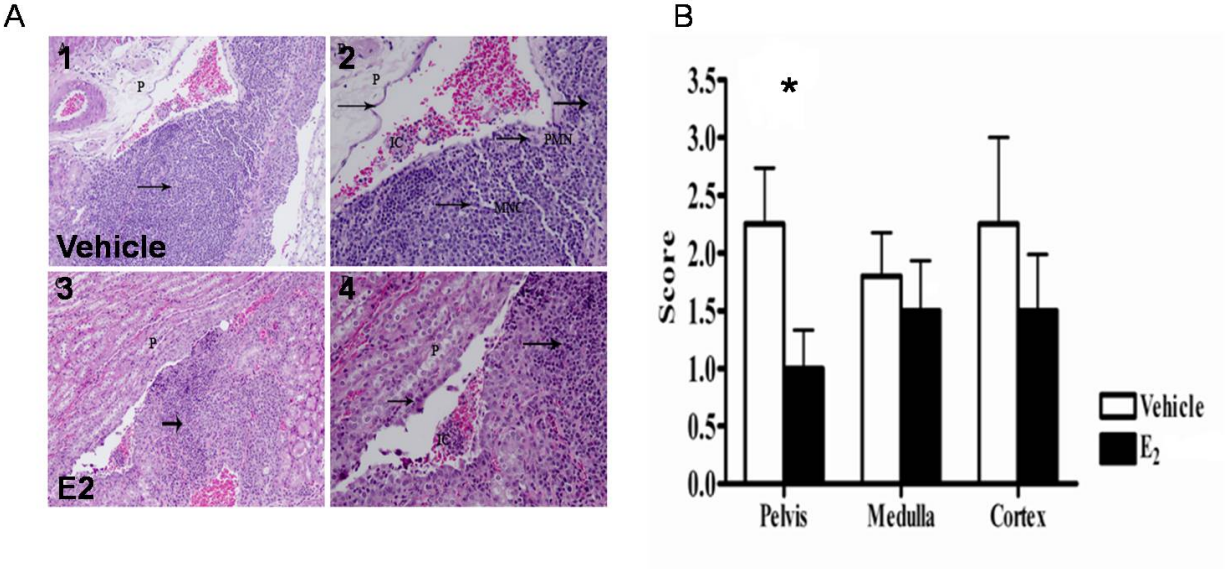


**Figure 2**

**Figure 1: Bacterial counts in the kidneys of C3H/HeJ mice at 21 days post-UTI induction.** OVX mice were treated with vehicle (Veh) or E2. The number of viable bacteria (CFU) from Non-OVX mice (n = 14) are depicted by filled circles while bacterial counts from vehicle (n = 28) and E2 (n = 27) treated OVX mice are indicated by closed triangles and open circles, respectively. Each symbol represents the number of viable bacteria from a single mouse. Bacterial counts are presented as mean CFU ± SEM and the error bars represent data from two independent experiments. Data were analyzed by One-way ANOVA, followed by Tukey's post hoc test for multiple comparisons where $P < 0.05$ was considered significant (*** indicate $P < 0.0001$). (Unpublished data)

**Figure 2: Representative renal pathology in C3H/HeJ mice at 21 days post-UTI induction**. **A)** Histopathological analysis of the kidney tissues from vehicle- (1, 2) or E2- (3, 4) treated groups of OVX mice, 21 days post-UTI induction. Images represent hematoxylin-eosin staining of kidney tissue sections. Arrows are pointing at the lymphocytes and neutrophils infiltration. **B)** The inflammatory scores taken from the pelvis, medulla and cortex of vehicle and E2 treated tissues are based on cellular infiltrate and fibrosis in the kidney tissues and are presented as mean score ± SEM. The error bars represent scores from 8 mice for each treatment group. Scores represent a scale of 0 to 4, where 0 represents normal or no inflammation and 4 represents end stage disease with chronic inflammation. Data were analyzed by Student's t test for each region in the kidney tissue section (pelvis, medulla and cortex) where $P < 0.05$ was considered significant (*). (Unpublished data)

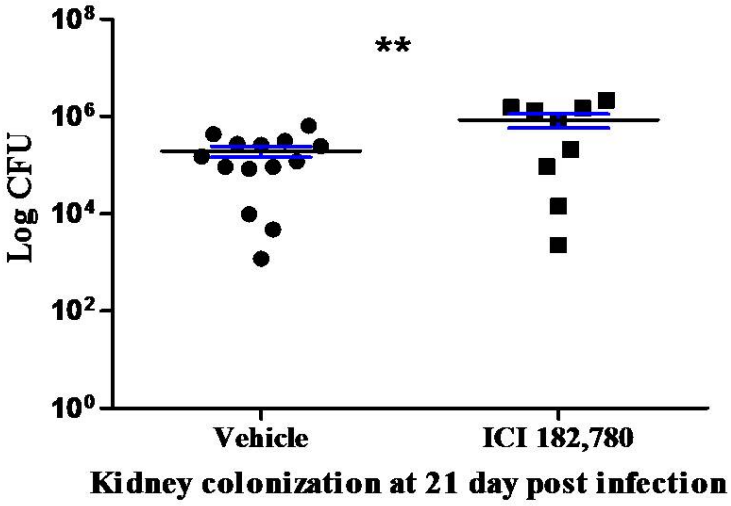


**Figure 3**

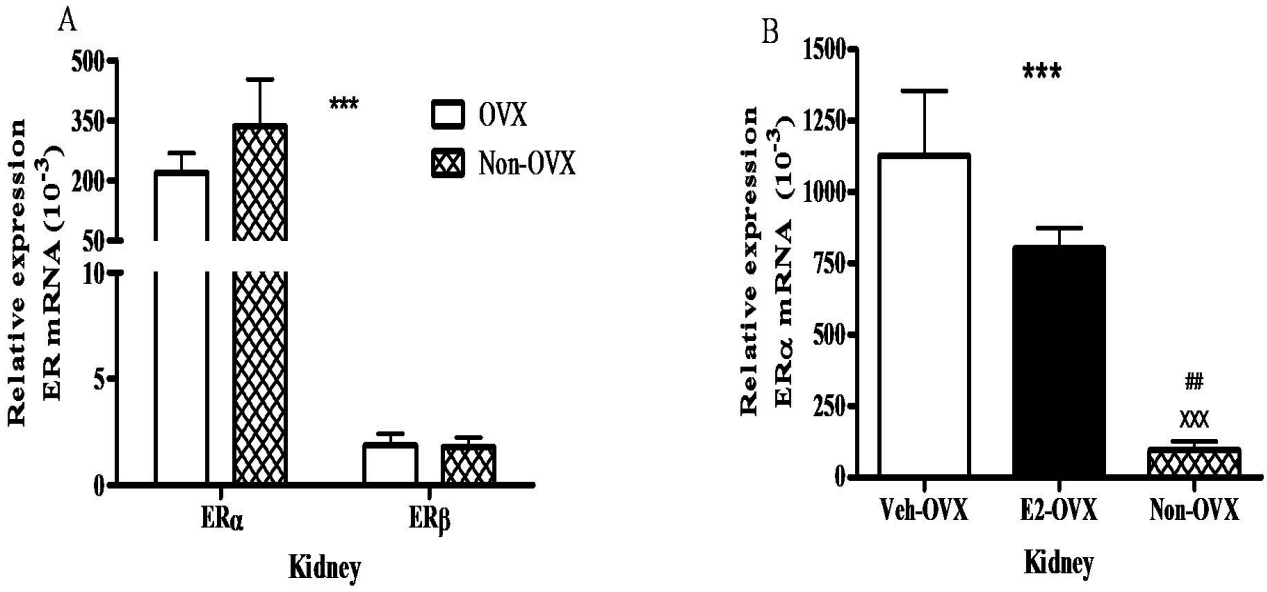


**Figure 4**

**Figure 3: Bacterial counts in the kidneys of ICI 182, 780-treated C3H/HeJ mice at 21 days post-UTI induction**. Non-OVX mice were treated with vehicle (Veh) or an ER-antagonist ICI 182, 780, prior to UTI induction. Viable bacterial counts from vehicle treated mice (n = 14) are depicted by filled circles, and bacterial counts from ICI 182,780 treated mice (n = 9) are indicated by closed squares. Bacterial counts from two independent experiments are presented as mean CFU ± SEM. The error bars represent data from n=9 and n=14 animals for vehicle and ICI 182, 780-treated group of animals, respectively. Data were analyzed by Student's t-test where $P < 0.05$ was considered significant (** indicate $P < 0.001$). (Unpublished lab data).

**Figure 4: ER subtype mRNA levels in the kidneys of C3H/HeJ mice. A)** The Kidney ERα and ERβ mRNA levels of uninfected, OVX and Non-OVX C3H/HeJ mice. **B)** The kidney ERα mRNA levels of Non-OVX C3H/HeJ mice and vehicle-(veh) or E2-treated OVX mice at 21days post-UTI induction. The mRNA levels were analyzed by SYBR green quantitative real-time RT-PCR. The expression levels of ER subtypes are normalized to PPIA expression. Expression values are presented as mean relative expression ± SEM. and the error bars represent data from 10-11 mice per group for Figure 4A and 4-6 mice per group for Figure 4B. Data were analyzed by One-way ANOVA followed by Tukey's post hoc test for multiple comparisons. $P< 0.05$ was considered significant (*** indicate $P < 0.000$). The character ## ($P < 0.001$) indicate significant difference compared to E2-OVX and xxx ($P < 0.0001$) indicate significant difference compared to Veh-OVX.

Following infection, the ERα mRNA levels in the estrogen-sufficient Non-OVX, and the E2-treated OVX mice were found to be markedly reduced compared to those in the estrogen-deficient- vehicle- treated OVX mice. Reduction in ER transcription level following activation with estrogen has been previously reported (Ihionkhan *et al*, 2002, Lee & Gorski, 1998). Reduced levels of ERα in estrogen-sufficient mice and increased levels of ERα in estrogen deficient mice following infection suggests that ERα activation may have an important functional role during Dr+ *E. coli* induced UTI pathogenesis (Figure 4B).

E2-mediated protection at physiological doses was also observed in our *in vitro* invasion studies using Huh7 hepatoma cells and in primary human hepatocytes. Primary human hepatocytes were obtained from University of Minnesota, NIH Liver Tissue Procurement and Distribution System (LTPADS) (PI collaboration). Human hepatocytes, as well as Huh7 cells, expressed DAF and both the ER subtypes. Pretreatment of cells with physiological doses of E2 (0.126ng/ml-1.26ng/ml) significantly reduced bacterial invasion in the Huh7 cells, as well as in the human hepatocytes (Figure 5). E2-mediated protection was significantly reversed in Huh7 cells when treated with ER- antagonists ICI 182,780 and 4-hydroxytamoxifen (OHT), suggesting involvement of ERs (data not shown).

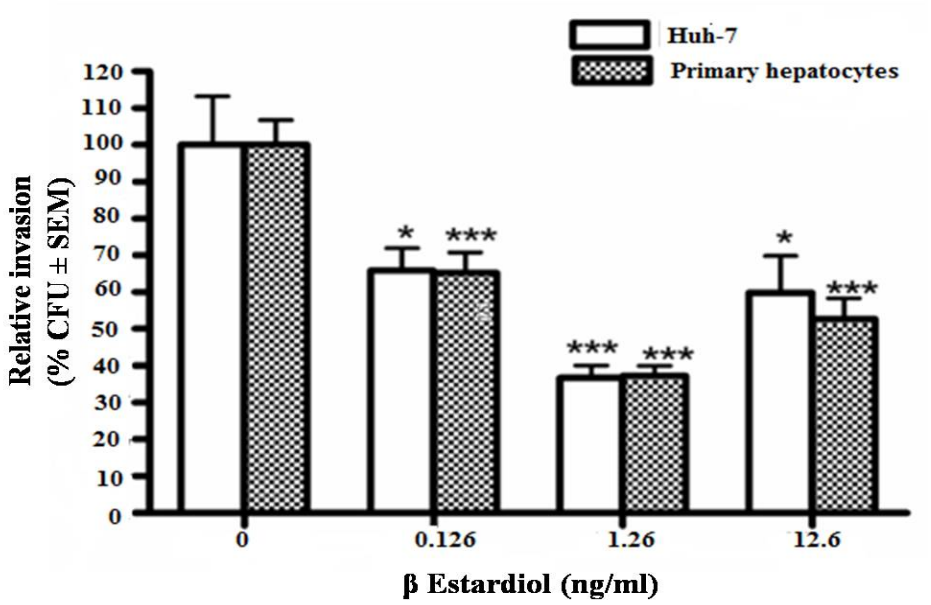


**Figure 5: Relative invasion of Dr+ *E. coli* in the E2-treated Huh7 cells and the primary human hepatocytes**. Relative bacterial invasion in cells treated with various doses of E2, where 1.26 ng/ml E2 corresponds to 5nM E2. The E2 doses ranging from 0.126-1.26 ng/ml are considered physiological. Invasion values are mean bacterial CFU ± SEM expressed as percentage of the bacteria internalized, relative to that in the untreated cells (invasion in control untreated cells was considered as 100 %). The error bars represent mean % bacterial CFU ± SEM from at least three independent experiments performed in triplicate, for each cell type. Data were analyzed by One-Way ANOVA for Huh7 and primary human hepatocytes followed by Tukey's post hoc test for multiple comparisons. $P < 0.05$ was considered significant. The character * indicate significant difference compared to the control in each cell type, where * indicate $P < 0.05$, **indicates $P < 0.005$, and *** indicates $P < 0.0005$. (Unpublished lab data).

## 1.3 Hypothesis

Based on the observations from our preliminary studies, we hypothesize that host endogenous estrogen levels, as well as ER subtypes expression, may play an important role in determining the host susceptibility to Dr+ *E. coli* mediated UTIs and associated inflammatory responses. We further hypothesize that estrogen via ERα activation may regulate the expression of host bacterial colonization receptors, DAF and type IV collagen, as well as activation of tyrosine kinase signaling pathway following Dr+ *E. coli* infection. In addition, we propose that estrogen regulates the inflammatory responses following Dr+ *E. coli* infection by modulating the expression of inflammatory markers, TLRs and pro-inflammatory cytokines.

## 1.4 Specific Aims

We tested our hypothesis using both, *in vivo* and *in vitro* studies as outlined in the following two specific aims.

**AIM-1:** *Elucidate the role of estrogen and ERα in regulation of* Dr+ *E. coli colonization and subsequent inflammation in the urinary tract of OVX C3H/HeJ mice during ascending UTI.*

The experimental UTI murine model established in OVX C3H/HeJ mice was used in the present *in vivo* experiments. C3H/HeJ mice carry a mutation in TLR4 that results in TLR4 signaling defect and LPS unresponsiveness in these mice (Poltorak *et al,* 1998).

Increased susceptibility to UTIs is reported in C3H/HeJ mouse and thus it serves as an appropriate model for inducing experimental UTIs (Hoshino *et al*, 1999, Poltorak *et al*, 1998).

**<u>Aim 1A:</u> Determine the effects of estrogen and ERs activation on UTI susceptibility by studying bacterial colonization in mouse kidney following Dr+ *E. coli* infection.**

The impact of estrogen deficiency and physiological E2 doses on UTI susceptibility was evaluated in vehicle- or E2-treated OVX C3H/HeJ mice induced with Dr+ *E.coli* -mediated experimental UTI. We evaluated the changes in bacterial colonization in the bladders and kidneys of these mice at 2 and 6 days post-infection. Impact of pure ER antagonist, ICI 182,780 or vehicle-treatment on UTI susceptibility in Non-OVX mice was determined to confirm the role of ERs in estrogen protective effects. Furthermore, UTI was induced in female ERα gene knock-out (ERα-/-) and ERα gene intact (ERα+/+) mice to confirm the role of ERα in mediating protective effects of estrogen against UTI susceptibility.

**<u>Aim 1B:</u> Determine the effects of estrogen and ERs action in regulating expression of Dr+ *E. coli* host colonization receptors and inflammatory markers in mouse kidney following infection.**

Expression of Dr+ *E. coli* colonization receptors, DAF and type IV collagen, inflammation inducers, TLR2 and TLR4, and pro-inflammatory cytokines, TNFα, IL6, and MIP2 was determined in the kidneys of mice at 2 and 6 days following

experimental UTI induction as described in Aim 1A. Expression analysis of target genes at the mRNA level was performed by quantitative SYBR green real time RT-PCR.

**AIM2:** *Establish an in vitro model of* Dr+ *E. coli infection in mouse kidney cell line, mIMCD3 cells to study E2/ER mediated modulation of* Dr+ *E. coli infection at the cellular level.*

The most distal renal tubule segment, the medullary collecting ducts are the first to come in contact with the ascending UPEC. They serve as the primary site for bacterial adherence and early inflammatory responses against UPEC (Chassin *et al*, 2006, Vandewalle, 2008). Therefore, we used a mouse kidney inner medullary collecting duct cell line (mIMCD3) for establishing our *in vitro* cell model for studying Dr+ *E. coli* induced uropathogenesis (Rauchman *et al*, 1993).

**<u>Aim 2A:</u> Determine the impact of E2 and ERs on Dr+ *E. coli* invasion and expression of Dr+ *E. coli* adhesin receptors and inflammatory markers in mIMCD3 cells following infection.**

Effects of pretreatment with E2, ER antagonist (ICI 182,780), an ERα selective agonist ( propyl pyrazole triol: PPT), an ERα selective antagonist (methyl piperidino pyrozole: MPP), an ERβ selective agonist ( diaryl propio nitrile: DPN) and an ERβ selective antagonist (R, R-tetrahydrochrysene: R,R-THC), on Dr+ *E. coli* invasion in mIMCD3 cells were determined. Changes in bacterial invasion and mRNA levels of

DAF, type IV collagen, TLR2, TLR4, TNFα, IL6, and MIP2 mRNA were analyzed following drug treatments in Dr+ *E. coli* infected, as well as uninfected mIMCD3 cells. Bacterial invasion was assessed by the standard gentamicin protection assay and mRNA levels were determined by quantitative SYBR green real-time RT- PCR.

**Aim 2B: Determine the impact of E2 on regulation of tyrosine kinase signaling pathway in the mIMCD3 following Dr+ *E. coli* infection.**

The impact of tyrosine kinase inhibitor, genistein and phosphoinostide 3-kinase (PI3K) inhibitor, LY294002 on bacterial invasion in mIMCD3 cells was determined to study the role of tyrosine kinase activation during Dr+ *E. coli* internalization in these cells. We also determined, the effect of E2 on activation status of tyrosine kinase in these cells was determined by detecting the levels of cellular signaling proteins, phosphorylated PI3K (pPI3K) and Akt (pAkt) (Protein kinase B) using western blots.

These specific aims, comprising of *in vivo* as well as *in vitro* studies showed that the molecular mechanisms associated with estrogen mediated attenuation of Dr+ *E. coli* mediated kidney infection involve ER mediated regulation of bacterial adherence receptors expression and host inflammatory responses. Furthermore, the *in vitro* mIMCD3 cell model of Dr+ *E. coli* uropathogenesis allowed us to demonstrate that Dr+ *E. coli* internalization in mIMCD3 cells require activation of tyrosine kinase involving PI3k/Akt signaling pathways and that estrogen differentially regulates the activation of these pathways, thus influencing bacterial invasion.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 UTI and UPEC

UTIs are the most frequently acquired bacterial infections, are considered to be the most common hospital acquired infections and are the second most common community acquired infections (Valiquette, 2001). UTIs cause significant morbidity and mortality in humans accounting for substantial healthcare costs (Foxman & Brown, 2003, Foxman *et al*, 2003, Foxman, 2003, Johnson & Russo, 2005). Females are significantly more likely to experience UTIs than men, and about 40-50 % of women are estimated to suffer from UTIs in their life time (Brown *et al*, 2005, Foxman *et al*, 2003, Franco, 2005, Zhang & Foxman, 2003). UTIs affect the upper as well as the lower urinary tract (Diagram 1) and include urethritis, cystitis, pyelonephritis, recurrent UTI, and asymptomatic bacteriuria. Pyelonephritis is the most severe form of UTIs, occurring in all age groups and gender with prevalence observed in females; about 10-30% of all patients with acute pyelonephritis are hospitalized for treatment (Brown *et al*, 2005). Incidence of UTI seems to vary with age and menstrual cycle, suggesting hormonal regulation of susceptibility to UTIs (Franco, 2005, Hooton, 2000, Ribeiro *et al*, 2002, Sonnex, 1998). Post-menopausal women have a high prevalence of UTI and estrogen

deficiency is considered to be an important contributing factor (Altoparlak *et al*, 2004, Foxman *et al*, 2001, Franco, 2005, Pabich *et al*, 2003).

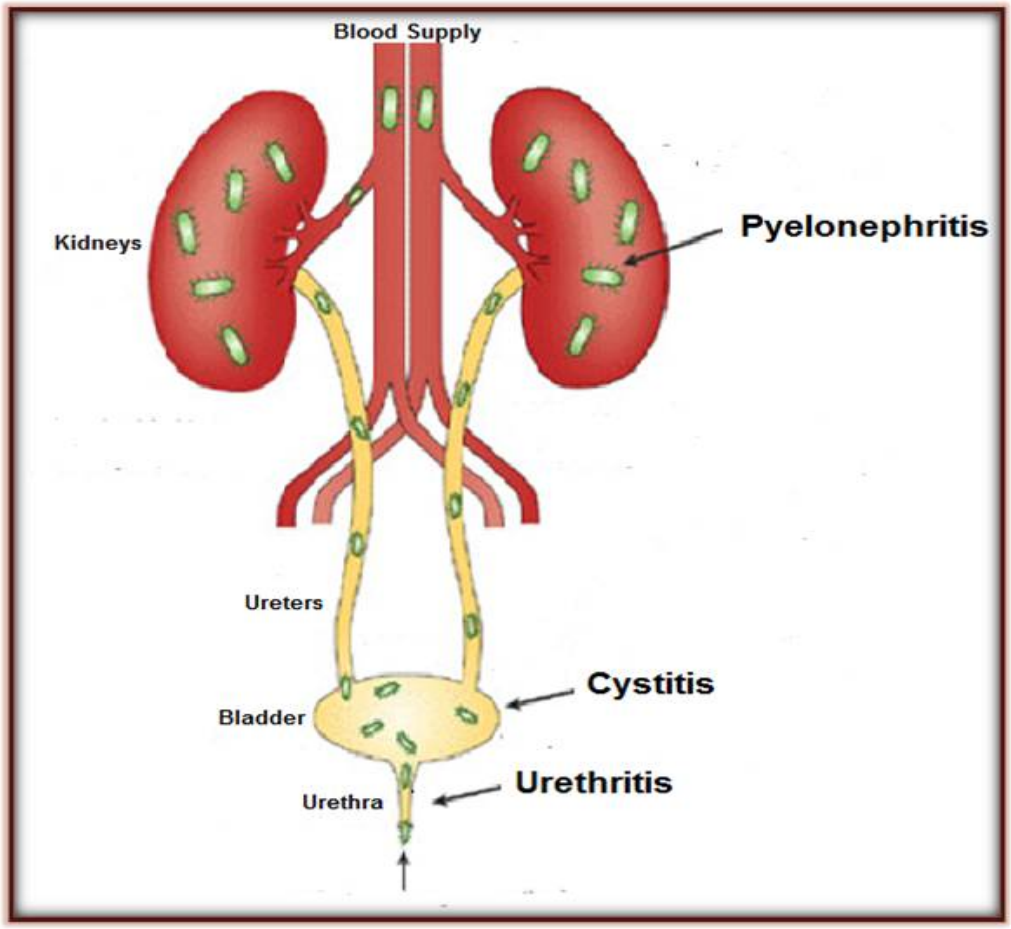


**Diagram 1: Sites of infection in the urinary tract.** Image adapted and modified from *Nat Rev.Microbiology,* 2, 2004 (Kaper et al, 2004)

UPEC are the most prevalent etiological agent of all UTIs including recurrent UTI, cystitis, and pyelonephritis (Johnson & Russo, 2002, Johnson, 2003, Ronald, 2002, Russo & Johnson, 2003, Zhang & Foxman, 2003). They are the primary cause of community acquired UTIs (70-95%) and many nosocomial UTIs (50%) (Foxman, 2003).

The urinary tract is considered to be a sterile site and entry of microbes into the urinary tract is mostly associated with physical introduction of organisms that colonize the perianal and periurethral areas. The gastrointestinal tract is thought to be the main reservoir for these uropathogens (Wiles *et al*, 2008). Commensal bacteria colonizing the gastrointestinal tract include several strains of *E. coli* that may inhabit this site for all of the individual's life, or only be present transiently. Several of these strains may colonize the periurethral areas and gain entry into the bladder via ascending route but may or may not cause any infection in the urinary tract. The *E. coli* strains which do persist and colonize the urinary tract but do not invade the uroepithelium or initiate any UTI symptoms show characteristics similar to the spectrum of gut colonizing *E. coli*. However, those bacteria who enter and elicit symptoms or cause asymptomatic UTI are not random samples of fecal *E. coli*; rather, they are a subset of *E. coli* strains known as the uropathogenic *E. coli* commonly referred as UPEC (Johnson & Russo, 2002, Johnson, 2003, Johnson & Russo, 2005, Marrs *et al*, 2005). These strains differ from fecal strains by possessing virulence factors unique to enable invasion and infection in the urinary tract and most of these virulence factors are coded by a stretch of chromosomal DNA called Pathogenicity Associated Islands (PAIs) (Bergsten *et al*, 2005, Bower *et al*, 2005, Brzuszkiewicz *et al*, 2006, Ronald, 2002, Yamamoto, 2007). In some cases the dissemination of a single clone group of a UPEC isolate may occur within a community via contaminated food or consumables (Manges *et al*, 2001, Manges *et al*, 2006, Manges *et al*, 2008). Aditionally, UPEC isolated from sexually active patients often matches with bacteria in the fecal isolates from their partners, indicating that UTI can be sexually transmitted (Foxman *et al*, 2002, Manges *et al*, 2004). UPEC strains act as opportunistic

intracellular pathogens taking advantage of host behavior and susceptibility by employing a diverse repertoire of virulence factors to colonize the urinary tract. The virulence factors include but are not limited to polysaccharide capsule (to evade phagocytosis), adhesion factors (to attach to the uroepithelium to prevent being washed away by urine flow), siderophores (to acquire iron), and cytotoxins (for invasion); these virulence factors facilitate the UPEC's ability to colonize and invade the uroepithelium (Johnson, 2003, Yamamoto, 2007).

UPEC adhesins are very crucial virulence factors as they allow the uropathogens to firmly adhere to the uroepithelium and initiate bacterial colonization in otherwise hostile environment (Antao *et al*, 2009, Wright & Hultgren, 2006). Adhesins of UPEC determine the bacterial affinity for and persistence in the urinary tract (Mulvey, 2002, Schilling *et al*, 2001a). Urinary tract colonization by virulent UPEC strains is mediated via various fimbrial and afimbrial adhesins including type I fimbriae, P pili, or Dr adhesins which determine the tissue specificity of UPEC. The uropathogens use these adhesive factors to firmly adhere to the receptors on the uroepithelium in the urethra and further ascend to the lower urinary tract (bladder) and upper urinary tract (kidney) establishing UTIs (Goluszko *et al*, 1997, Hagberg *et al*, 1983b, Hagberg *et al*, 1983b, Johnson & Russo, 2005, Nowicki *et al*, 2001, Svanborg & Godaly, 1997). In an experiment studying the ascent of bacteria from the bladders to the kidneys following bladder catheterization in mice, it was found that <0.01% of the inoculum reaches the kidneys (Hagberg *et al*, 1983a). In the urinary tract a thin film of fluid around the luminal surface of the epithelium represents the 'battlefield' where uropathogen interact

with the epithelial cells and the innate immune system, resulting in bacterial invasion and inflammation that finally contribute to uropathogenesis and cellular damage (Diagram 2) following UPEC infection (Brogden *et al,* 2005, Zasloff, 2006, Zasloff, 2007).

UPEC strains may ascend to the lower and the upper urinary tract via uroepithelial binding sites. Like enteric *E. coli* pathogens, UPEC isolates are genetically heterogeneous and vary significantly in their abilities to colonize and persist within either the bladder or the kidney (Mulvey, 2002). For example, the pyelonephritogenic P fimbriated *E. coli* colonize the apical surface of the renal tubules via attaching to the α D-Gal-(1,4)-β-D-Gal containing surface glycolipid receptors present abundantly in the kidney. In contrast, the Type 1 pili bearing UPEC that bind to the mannosylated glycoproteins are significantly associated with the bladder infection. Less well characterized S pili bearing UPEC are associated with cystitis, pyelonephritis and may cause disseminated infection including sepsis and meningitis. The S pilus has been found to attach to the sialic acid residues of the receptors expressed on kidney, bladder and endothelial cells. The F1C pili can bind to β-GalNac-1, 4β-Gal residues on glycolipids expressed by epithelial cells of the distal tubules, collecting ducts of the kidney, bladder and kidney endothelial cells, and may contribute significantly to the pathogenesis of a number of UTI cases. Furthermore, the Dr adhesin bearing UPEC that attach via complement regulatory receptor DAF/CD55 (expressed on the erythrocytes,

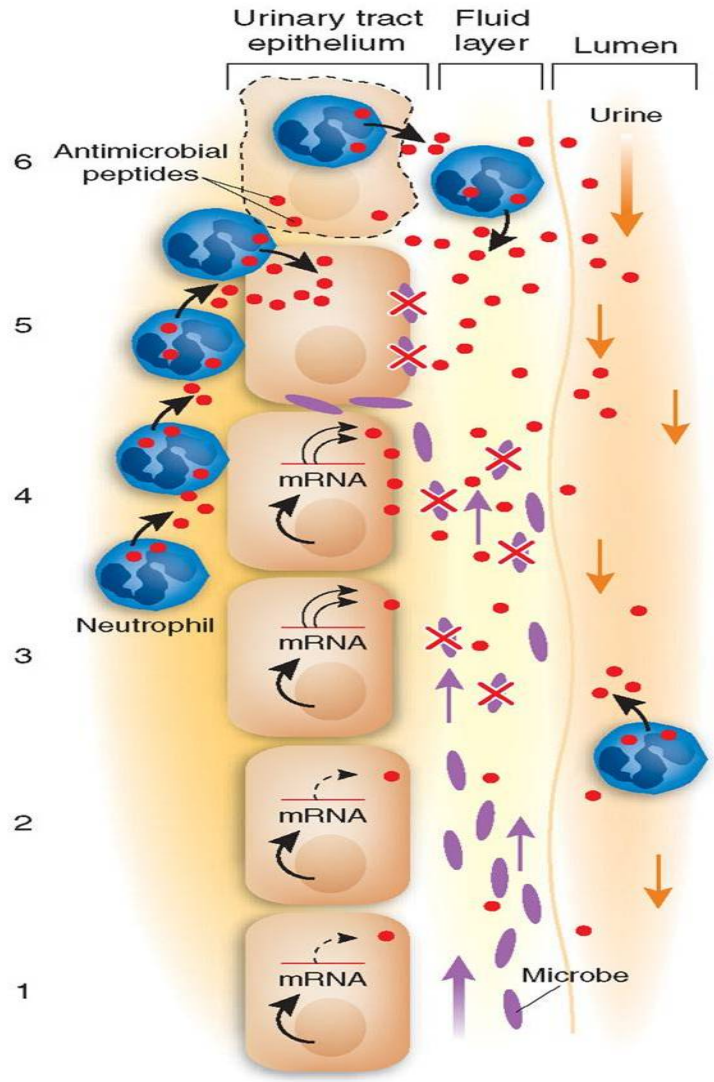


**Diagram 2: Uropathogen and host interaction during establishment of infection in the urinary tract by ascending UPEC**. **1**, Uroepithelium constitutively express and secrete antibacterial peptides in the lumen of urinary tract ; **2**, UPEC ascend and approach the epithelial surface anticipating attachment; **3**, antibacterial peptides levels rapidly rise, translation is activated, and peptide secreted rapidly, killing nearby bacteria, some UPEC survive and continue to ascend; **4,** neutrophils begin to migrate toward the lumen; **5,** neutrophils secrete antibacterial peptides, complementing epithelial sources, but viable microbes persist; **6** neutrophils continue to invade the epithelium and so does the uropathogen, resulting in cellular damage. Image adapted and modified from *Nature Medicine*, 12, 2006 (Zasloff, 2006).

endothelial, and epithelial cells) are associated with recurrent UTI, 30-50% of cystitis cases and about 30% of pregnancy associated pyelonephritis cases (Servin, 2005)

## 2.2 Uropathogenic Dr+ *E. coli*

Dr+ adhesin bearing *E. coli* belong to the family of Afa/ Dr adhesin bearing *E. coli* broadly classified as diffusely adhering *E. coli* (DAEC). Afa/ Dr DAEC strains exhibit identical genetic organization, they bind to human DAF/CD55 and cause intestinal (diarrhea) as well as extraintestinal (uropathogenic) infections (Servin, 2005). Uropathogenic Dr+ *E. coli*, strain IH11128 is associated with recurrent UTI, cystitis, and pyelonephritis in humans (Goluszko *et al*, 1997, Goluszko *et al*, 2001, Nowicki *et al*, 2001, Servin, 2005). It has been suggested that first UTI infection with Dr+ *E. coli* increases the risk for second UTI in patients by 2-fold (Foxman *et al*, 1995, Zhang & Foxman, 2003). Adherence to host epithelium by UPEC is crucial for establishing infection. Dr adhesin provides attachment of the Dr+ *E. coli* to the luminal apical surface within the urinary tract. An isogenic Dr negative mutant (Dr14/ Dr- *E. coli*) of IH11128 was developed by insertional inactivation of *draC* gene of Dr+ *E. coli* (Goluszko *et al*, 1997). The *draC* gene product is essential for the assembly of functional Dr adhesin, thus Dr14 *E. coli* do not assemble functional Dr adhesins and show reduced adherence and virulence towards the host cells (Goluszko *et al*, 1997, Kaul *et al*, 1999). This confirms that Dr adhesin of Dr+ *E. coli* mediates bacterial adherence to host epithelial cells and is important for host colonization. Dr+ *E. coli* has been successfully used for developing experimental chronic pyelonephritis in C3H/HeJ mouse model where Dr adhesin was found to be critical for development of chronic pyelonephritis (Goluszko *et*

*al*, 1997, Nowicki *et al*, 2001). Dr adhesin was found to recognize a tyrosine containing molecule in the Dr blood group antigen that was further identified as the complement regulatory protein CD55 most commonly known as DAF. DAF is a membrane associated phosphatidyl-inositol anchored glycoprotein (GPI) expressed on all the blood cells, endothelial and epithelial cell surfaces. Dr adhesins bind at short consensus repeat (SCR)-3 and SCR-4 domain of DAF (Hasan *et al*, 2002, Nowicki *et al*, 1990, Nowicki *et al*, 1993, Nowicki *et al*, 2001, Pham *et al*, 1995, Selvarangan *et al*, 2000). Within the urinary tract, the epithelial surfaces of urethra, bladder, ureter, and renal pelvis are rich in DAF expression and bind to Dr fimbria (Nowicki *et al*, 1988). Dr adhesins of Dr+ *E. coli* also recognize type IV collagen as its colonization receptor in the host urinary tract (Selvarangan *et al*, 2004, Westerlund *et al*, 1989). Type IV collagen is an important component of the specialized extracellular matrix (ECM) that underlies all the epithelial membrane and functions to compartmentalize the tissue. Dr adesins binds to the renal tubular basement membrane and the Bowman's capsule in the renal glomerulus which have abundant type IV collagen (Nowicki *et al*, 1988). Dr+ *E. coli* recognizes DAF/CD55 to adhere, invade, and colonize the kidney epithelium (Nowicki *et al*, 2001, Selvarangan *et al*, 2000, Selvarangan *et al*, 2004, Servin, 2005) and further binds to type IV collagen in the epithelial basement membrane which allows it to localize in the interstitial compartment of the renal tissues (Diagram 3) that may further contribute to pyelonephritis (Selvarangan *et al*, 2004, Mulvey, 2002).

Interaction of Dr+ *E. coli* with DAF and type IV collagen in the kidney leads to bacterial infection and persistence inducing inflammation in renal tubules and

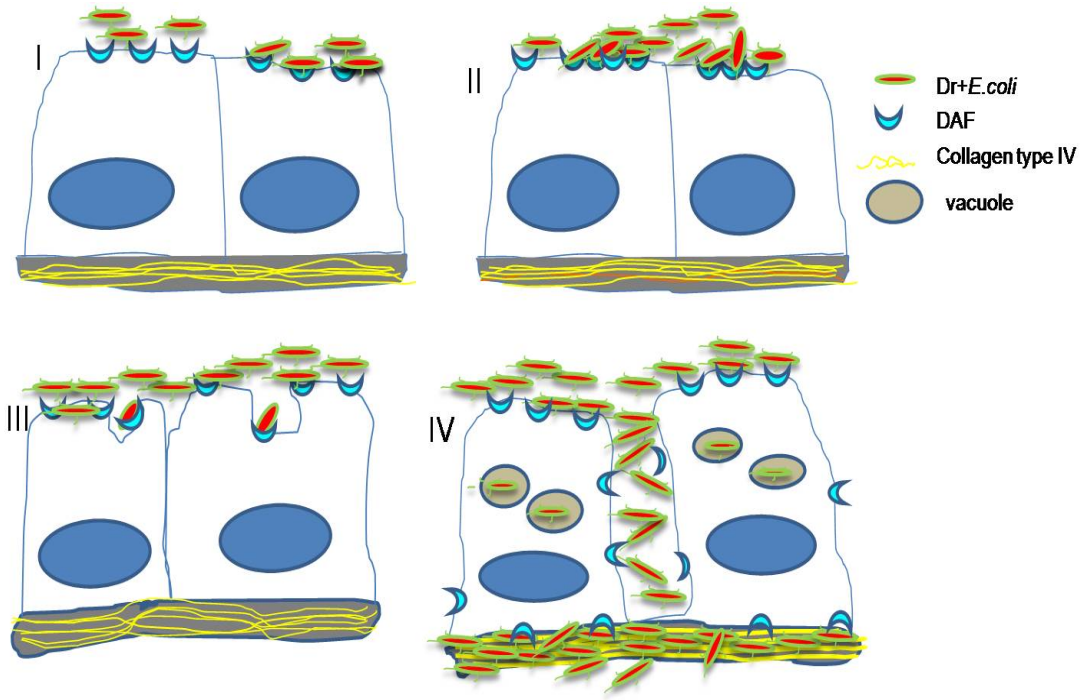


**Diagram 3: Mechanism of Dr+ *E. coli* uropathogenesis**. **I,** Bacterial colonization and attachment to DAF on the apical surface of the host renal epithelium; **II**, clustering of DAF following bacterial attachment; **III**, Initiation of bacterial internalization by phagocytic pathway and introduction of lesion at the tight junction on the epithelial surface; **IV**, Bacterial internalization into a vacuole within the host cell, loss of epithelial polarization, invasion of bacteria via paracellular route and attachment of bacteria to type IV collagen in the basement membrane of the epithelium facilitating renal persistence of Dr+ *E. coli* and development of pyelonephritis. Image based on information published in *Cell Microbiology*, 4, 2002 (Mulvey, 2002)

interstitium. In an *in vitro* cell monolayer model, Dr+ *E. coli* were shown to increase DAF expression and IL-8 production leading to increased transepithelial neutrophil migration, suggesting possible mechanism of increased adherence and inflammatory activation during Dr+ *E. coli* pathogenesis (Betis *et al*, 2003a, Betis *et al*, 2003b). Dr+ *E. coli* are invasive bacteria that bind to GPI-DAF on epithelial cell surface subsequently leading to their internalization by the cells (Diagram 3) (Goluszko *et al*, 1997, Kansau *et al*, 2004, Selvarangan *et al*, 2000, Mulvey, 2002). Recruitment of DAF and α5β1integrins around Dr+ *E. coli*, following the bacterial engagement is crucial for bacterial internalization (Das *et al*, 2005, Goluszko *et al*, 1999, Guignot *et al*, 2001, Kansau *et al*, 2004, Plancon *et al*, 2003, Selvarangan *et al*, 2000). The cellular invasion by Dr+ *E. coli* has been shown to occur via a zipper like mechanism (Diagram 4) employing accumulation of polymerized actin around the adherent bacteria followed by microtubule mediated internalization of the bacteria containing vacuole into the cell (Das *et al*, 2005, Goluszko *et al*, 1999, Guignot *et al*, 2001, Kansau *et al*, 2004).

Internalization of *E. coli* by renal epithelial cells is associated with tyrosine kinase mediated tyrosine phosphorylation of specific host proteins (Guignot *et al*, 2001, Palmer *et al*, 1997, Peiffer *et al*, 1998) (Diagram 5). DAF and integrins are known to activate tyrosine kinase following engagement with their ligands (Abedi & Zachary, 1997, Burridge *et al*, 1988, Shenoy-Scaria *et al*, 1992, Stefanova *et al*, 1991, Zachary & Rozengurt, 1992). PI3K has been identified as a downstream effector of both receptor and non-receptor tyrosine kinases (Wymann & Pirola, 1998). PI3K is a heterodimeric protein consisting of a regulatory subunit (p85) and a catalytic subunit (p110).

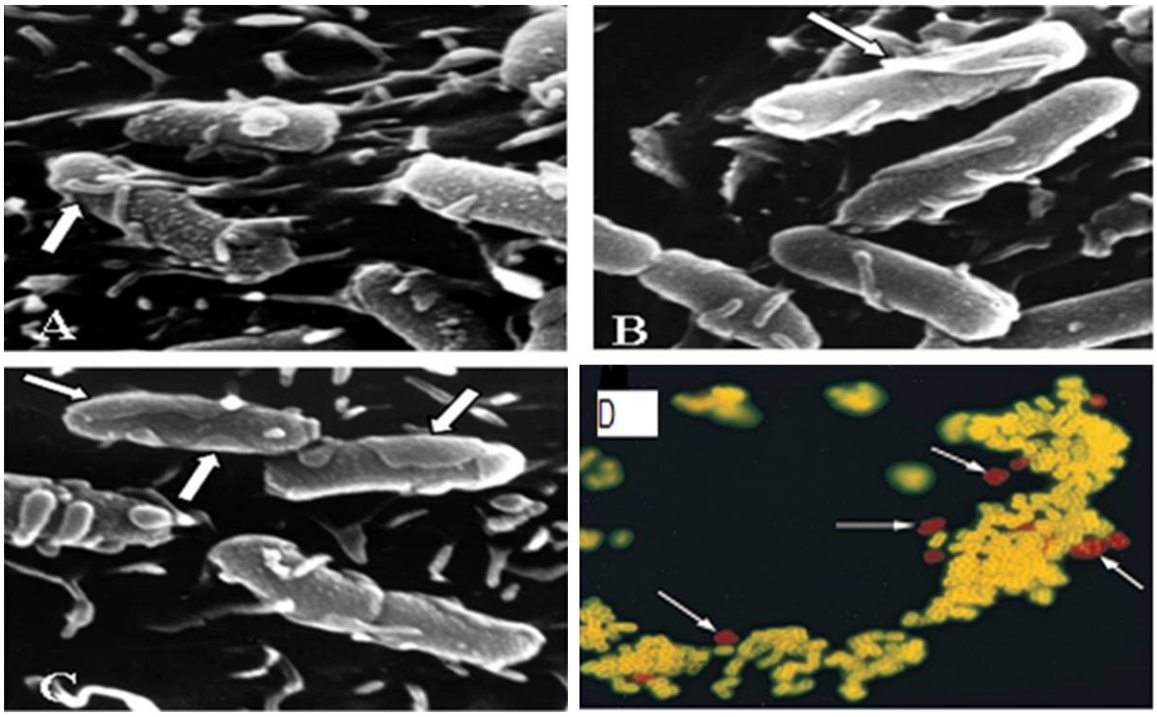


**Diagram 4: Dr+ *E. coli* internalization in the host epithelial cells.** Scanning electron microscopic examination of the zipper like internalization of Dr+ IH11128 *E. coli* into HeLa cells (A-C). **(A**) Microvillus-like extensions (arrow) extend from the cell surface and are associated with bacteria, **(B)** Fused extensions (arrow) forming a zipper-like structure, **(C)** Enlarged extensions (arrows) form a zipper-like structure. Image adapted from *Infection And Immunity*, 72, 2004 (Kansau *et al*, 2004). **D)** Dual immunofluorescence labeling of IH11128 bacteria in infected Caco-2/TC7 cells and visualization of extracellular and internalized bacteria with FITC or tetramethyl rhodamine isothiocyanate immunolabeling, respectively. Green extracellular bacteria and red intracellular bacteria (arrows) were seen at the periphery of the cell clusterin which proliferative cells were localized. Image adapted from: *Infection And Immunity*, 69, 2001 (Guignot *et al*, 2001).

The regulatory subunit of PI3K interacts with several intracellular signaling molecules. Interaction of p85 with phosphotyrosine residues on tyrosine kinase results in PI3K activation causing the catalytic subunit to phosphorylate the inositide ring of phosphotidylinositol to form phosphotidylinositol 3, 4, 5 tripohosphate (PIP3). PIP3 can then causes downstream activation of Akt (also known as protein kinase B-PKB). PI3K signaling has been implicated in a variety of cellular processes, including survival, proliferation, migration, metabolic changes and bacterial invasion (Diagram 5) into the cell (Brumell & Grinstein, 2003, Wymann & Pirola, 1998). PI3K has been shown to be necessary for the invasion of epithelial cells by several bacteria, including *Listeria monocytogenes* (Ireton *et al*, 1996), *Helicobacter pylori* (Kwok *et al*, 2002), *Neisseria gonorrhoeae* (Booth *et al*, 2003), *Pseudomonas aeruginosa* (Kierbel *et al*, 2005), Group B streptococci (Goluszko *et al*, 2008) and *Escherichia coli* K1 (Reddy *et al*, 2000). Dr adhesin expressing recombinant bacteria have been shown to require PI3K activity for internalization into primary human bladder epithelial cells which express DAF and carcinoembryonic antigen (CEA)-related cell adhesion molecules (CEACAMs) as receptors for Dr adhesins (Korotkova *et al*, 2008).

## 2.3 UTI and Inflammation

Pathology associated with UTI is caused due to UPEC mediated and inflammation related tissue damage. Pyelonephritis is associated with inflammatory damage of the pelvic and kidney tubules (Scherberich & Hartinger, 2007). UPEC attach to specific receptors through their adhesisn on host mucosa and trigger innate immune responses via

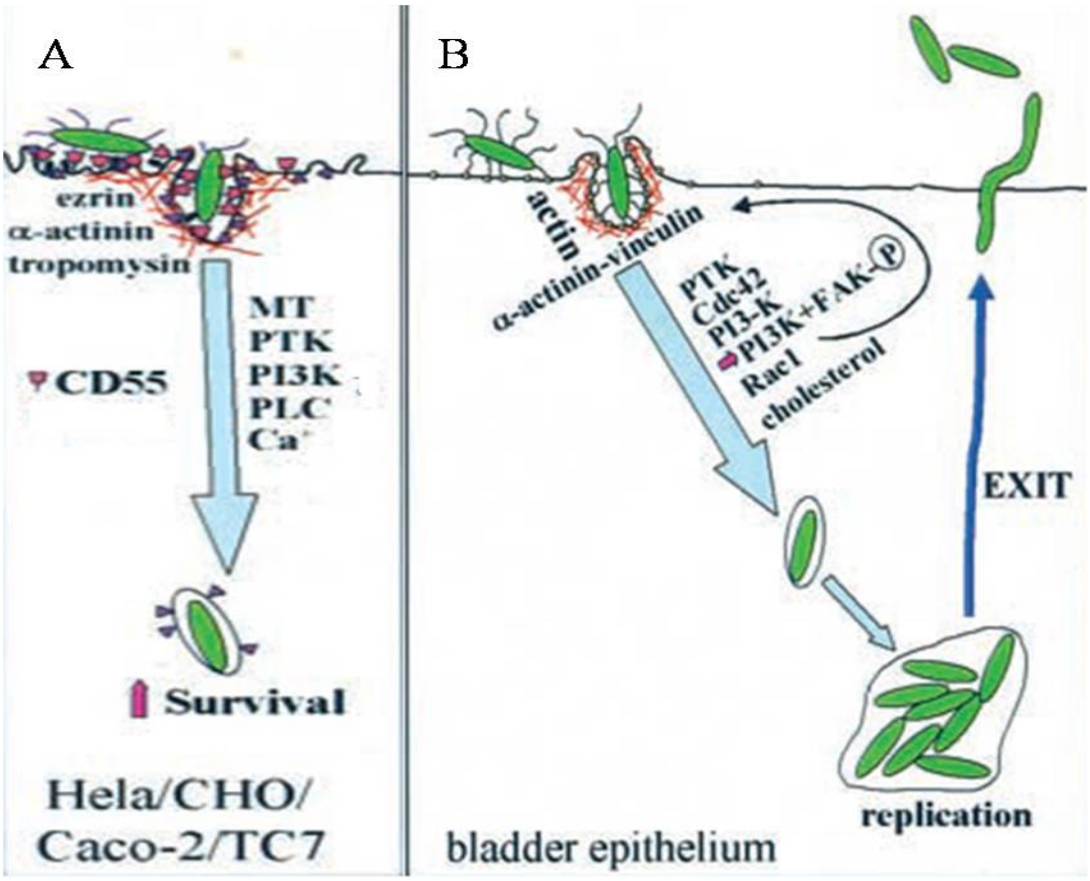


**Diagram 5: Mechanism of UPEC invasion in the host epithelial cells**. Cellular signaling molecules and host components involved in the internalization of UPEC studied in different cell types are outlined: A) Dr adhesin mediated entry into host epithelial cells and B) Type 1 pilus mediated entry into bladder epithelial cells. Following bacterial interaction with the cellular receptor for UPEC adhesisns, signal transduction cascades are activated leading to internalization of the bacteria. CD55/ DAF (Dr adhesisn receptor) PTK, protein tyrosine kinase; PI3-K, phosphoinositide-3-kinase; FAK, focal adhesion kinase; PKC, protein kinase C; PLC phospholopase Cγ and MT, microtubule. Image adapted and modified from *Cellular Microbiology*, 4, 2002 (Mulvey, 2002)

interactions with co-receptors including TLRs. Tissue damage during UTI is associated with UPEC mediated apoptosis in urothelium and activation of macrophages and neutrophils which contributes to renal cell apoptosis directly as well as indirectly (Billips *et al*, 2008, Kipari & Hughes, 2002, Klumpp *et al*, 2006, Lange-Sperandio *et al*, 2003). Epithelial cells are known to be the early producers of pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-1β, IL-8, RANTES, MCP-1) that are found to be increased in UTI patients and in experimental UTI mouse models (Agace *et al*, 1993, Betis *et al*, 2003a, Connell *et al*, 1997, Godaly *et al*, 2000, Hedges *et al*, 1994, Samuelsson *et al*, 2004, Scherberich & Hartinger, 2007, Svanborg *et al*, 1994, Svanborg *et al*, 1996, Svanborg *et al*, 1999, Wullt *et al*, 2002). During bacterial infection virulence factors are released and the immune system is activated to secrete pro-inflammatory cytokines. Induction of the inflammatory immune response subsequent to infection determines the course of disease and extent of tissue damage. An initial rapid inflammatory response is essential for clearing up the invading pathogen; however persistent chronic inflammation leads to tissue damage. Less virulent strains have been suggested to cause asymptomatic bacteriuria, where the bacteria is able to colonize the urinary tract without activation of inflammatory response and thus no symptoms associated with UTI is observed (Zdziarski *et al*, 2008). This suggests that inflammatory responses contribute to not only bacterial clearance, but also to tissue damage and associated symptoms during UTI, acting as a double edge sword.

Initial recognition of unique molecular patterns associated with pathogens by immune cells and epithelial cells via pattern recognition receptors like TLRs and other pattern

recognition receptors (PRRs) like -NOD like helicases and RIG like helicases- is the most crucial step in immune activation. Members of the TLR family are located on the plasma membrane and membranes of endogenous vesicles in renal epithelial and immune cells; they bind to different signature ligands from a wide variety of pathogens (Anders & Schlondorff, 2007, Anders, 2007, Vandewalle, 2008). Upon ligand binding and activation, these receptors interact with several adaptor proteins via their cytoplasmic domain leading to activation of cellular signaling pathways including NF-κB and MAP kinase (p38 and JNK). This response leads to up-regulation of cytokines, chemokines and antimicrobial products synthesis that are crucial for inflammation and bacterial clearance (Akira *et al*, 2006, Kaisho & Akira, 2006, Kawai & Akira, 2005a, Kawai & Akira, 2005b, Kawai & Akira, 2007, Li & Cherayil, 2004). For instance, secreations of CXC chemokines MIP2 (mouse) or IL-8 (humans) by renal epithelial cells upon TLR activation induce influx and activation of neutrophils in the renal interstitium, thus promoting interstitial inflammation (Samuelsson *et al*, 2004, Tang *et al*, 2003). Furthermore, reduced expression of IL8 receptors is associated with increased susceptibility to acute pyelonephritis (Lundstedt *et al*, 2007, Svensson *et al*, 2008). IL-6 production during UTI is associated with stimulation of IgA secretion by B cells contributing to protection against UTI (Hedges *et al*, 1991, Hedges *et al*, 1992). MCP-1 and RANTES are also reported to be secreted by renal tubular epithelial cells following TLR activation by bacterial components (Tsuboi *et al*, 2002). IFN-gamma and TNF-alpha released by activated renal tubule epithelium cells are responsible for regulating TLR2 and TLR4 expression in this tissue (Wolfs *et al*, 2002).

The UPEC interact with urethral, bladder epithelium and renal tubule cells upon infection in the urinary tract. The inner medullary collecting duct (IMDC) cells of the kidney are the first to come in contact with ascending UPEC strains, therefore they form the site for bacterial adherence and initiation of immune responses in the kidney (Chassin *et al*, 2006, Vandewalle, 2008). *In vivo* experiments with pyelonephritis murine models have demonstrated that UPEC adhered preferentially and constantly to the apical membranes of the medullary collecting duct cells following transurethral inoculation and induce expression and bipolarized secretion of MIP-2 via TLR4 dependent and independent pathways (Chassin *et al*, 2006). Renal tubular epithelial cells (Diagram 6) and interstitial myeloid dendritic cells are reported to constitutively express TLR1, 2, 3, 4, 5, and 6, and show enhanced cytokine production following UPEC interaction (Anders, 2007, Anders, 2007, Lech *et al*, 2007, Patole *et al*, 2005, Samuelsson *et al*, 2004, Scherberich & Hartinger, 2007, Svanborg *et al*, 2001b, Tsuboi *et al*, 2002, Vandewalle, 2008). TLR2 and TLR4 expressed on renal tubular cells have been shown to mediate direct inflammatory responses to bacterial products by activating secretion of cytokines and chemokines secretions (Chowdhury *et al*, 2006, Scherberich & Hartinger, 2007, Tsuboi *et al*, 2002, Wolfs *et al*, 2002). In mouse, TLR11 expressed on kidney epithelial cells is reported to play an important role in protection from uropathogenic bacterial infection (Zhang *et al*, 2004). LPS is the prominent gram negative *E. coli* product that activates TLR4 in immune and epithelial cells inducing proinflammatory immune responses. Besides LPS, UPEC produce various fimbrial and afimbrial adhesins that trigger TLR mediated inflammatory immune responses in the urinary tract independently of LPS

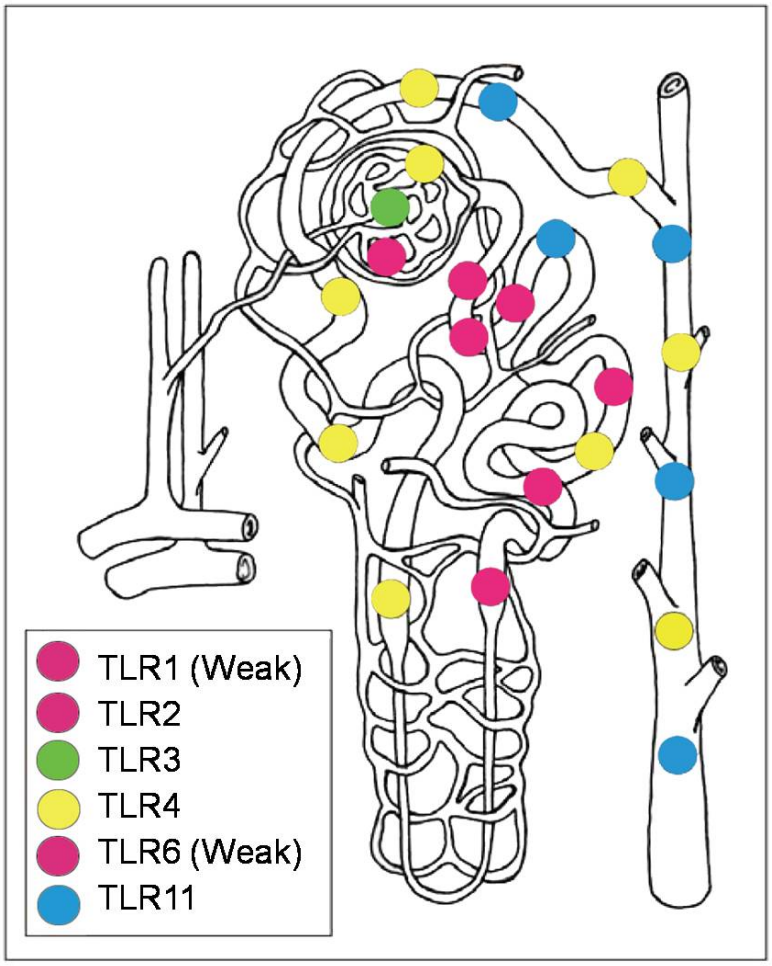


**Diagram 6: Intrarenal distribution of TLRs along the kidney nephrons**. Image adapted from *Chang Gunj Med J* , 31, 2008 (Vandewalle, 2008)

(Chassin *et al*, 2006, Fischer *et al*, 2006, Patole *et al*, 2005, Uhlen *et al*, 2000). In studies utilizing human kidney biopsies and an experimental murine UTI model, UPEC adhesins-P pili and type 1 fimbriae have been shown to initiate a rapid inflammatory response via TLR4 activation independent of LPS (Bergsten *et al*, 2007, Fischer *et al*, 2006, Fischer *et al*, 2007, Frendeus *et al*, 2001, Godaly *et al*, 2007, Samuelsson *et al*, 2004). Cirl *et al* have reported that UPEC strains promote bacterial survival and kidney damage by secreting products that interact and interfere with TLR activation (Cirl *et al*, 2008) suggesting importance of TLR signaling in the host defense against bacterial infection and is a possible virulence mechanism developed by pathogens to avoid TLR activation.

Reduction in TLR4 expression has been associated with asymptomatic bacteriuria (Ragnarsdottir *et al*, 2007). C3H/HeJ mice carry a point mutation in the cytoplasmic region of TLR4, a replacement of proline with histidine, causing defective signaling leading to the LPS hyporesponsive phenotype and increase susceptibility to bacterial infections (Hoshino *et al*, 1999, Poltorak *et al*, 1998). In C3H/HeJ mice this inactivating mutation also subdues the cytokine production in response to TLR2 mediated signaling (Li & Cherayil, 2004) suggesting that TLR4 activation is important for TLR 2 mediated inflammatory signaling. The attenuation of bacterial defense due to TLR4 polymorphism might become crucial during severe infections. In humans, Asp299Gly polymorphism of TLR4 is associated with defective LPS signaling (Agnese *et al*, 2002, Arbour *et al*, 2000, Lorenz *et al*, 2002), and the frequency of the Asp299Gly polymorphism of TLR4 ranges from 3% to 11% in the white human population (von Aulock *et al*, 2003). Recent studies

have shown a higher prevalence of gram-negative infections and a more severe course of disease for carriers of the Asp299Gly allele (Agnese *et al*, 2002, Arbour *et al*, 2000, Lorenz *et al*, 2002). Forthermore, studies in women with UTI have reported that adult susceptibility to UTI is associated with polymorphism in TLR2, TLR5 and TLR1gene (Hawn *et al*, 2009a, Hawn *et al*, 2009b). TLR5 that recognizes bacterial flagellin is an important inducer of inflammation in experimental UTI in mice and TLR5 gene knock-out mice were found to have increased susceptibility to UTI (Andersen-Nissen *et al*, 2007). In an experimental UTI murine model, presence of TLR 11 in the urinary tract was found to provide significant protection against uropathogen infection (Zhang *et al*, 2004). Thus, it is evident that TLR activation and cytokines secretion is critical for clearing UPEC from the host urinary tract. Moreover, with the rising trends in antibiotic resistant UPEC strains contributing to more frequent, recurrent, and chronic UTIs, it seems important to explore the possible immunomodulatory mechanisms as effective prophylaxis and treatment options for UTI patients with normal as well as dysfunctional immune system.

## 2.4 Estrogen and Estrogen receptors

Estrogen is a steroid hormone that acts via activation of its receptors ERα and ERβ. ERα and ERβ belong to the superfamily of nuclear receptors and specifically to the family of steroid receptors that act as ligand-regulated transcription factors. ERα and ERβ show high sequence homology and share affinity for the same ligands and DNA response elements. Estrogen is the biological ligand to both the ER subtypes subtypes; however ERα and ERβ exhibit different affinities for some natural and synthetic

compounds. They have distinct expression patterns in a variety of tissues and in different cell types, thus generating diverse effects following ligand induced activation (Barkhem *et al*, 2004, Barkhem *et al*, 2004, Nilsson *et al*, 2001, Nilsson & Gustafsson, 2002).

ERs consist of a C-terminal steroid ligand–binding domain (LBD), a centrally located DNA binding domain (DBD), and an N-terminal domain of less well-characterized function. Additionally, the ERs contain two autonomous transcriptional activation domains (AF); the AF-1 domain, located in the N terminus, and AF-2, located within the LBD. Transcriptional activation by ERα is mediated by two distinct activation functions: the constitutively active AF-1 and the ligand-dependent AF-2. ERβ seems to have a weaker corresponding AF-1 function and thus depends more on the AF-2 for its transcriptional activation function. ERs are latent transcriptional activators that require ligand binding for activation. The estrogen binding induces a specific conformational change(s) in the ERs resulting in dissociation of the receptors from a protein chaperone complex, dimerization of the ERs, and binding of the receptor dimer to the specific estrogen response elements (EREs) located in the 5′ regulatory region of the primary steroid-responsive target genes. Through interaction with AF-1 or AF-2, the activated, DNA-bound receptors recruit transcriptional co-activators that mediate assembly of productive transcription complexes at the promoter of target genes. ERα and ERβ have shown different activities in certain ligand, cell-type, and promoter contexts (Barkhem *et al*, 2004, Nilsson *et al*, 2001, Nilsson & Gustafsson, 2002).

Estrogen activated ERs regulate (enhance or repress) target gene expression either directly via the classical genomic pathway or indirectly via a rapid non-genomic signaling pathway (diagram 7). In the classical genomic pathway, the ligand activated ERs directly bind to estrogen responsive elements (ERE) in the target gene promoter or interact with other transcription factors involved in transcription modulation. In the non-genomic pathway, the estrogen activated ERs interact with membrane bound or cytoplasmic signaling molecules to modulate gene expression and cellular function (Katzenellenbogen, 1996, Katzenellenbogen *et al*, 2000, Losel & Wehling, 2003, McKenna & O'Malley, 2002). Although the classical pathway is considered the major pathway for estrogen action in transcriptional regulation of specific gene networks, it is now well understood that integration of membrane-initiated non-genomic signaling along with genomic mechanisms represents in the complete cellular response to estrogen in the target cells (Cato *et al*, 2002, Edwards & Boonyaratanakornkit, 2003, Watson & Gametchu, 1999).

## 2.5 Estrogen and Kidney

The kidney is considered to have the third largest number of estrogen regulated genes, and these genes are suggested to be regulated via ERα mediated mechanism (Jelinsky *et al*, 2003, Kuiper *et al*, 1997). Female mice were found to show more ERα than ERβ expression in renal cortex cells; ovariectomy associated with decreased estrogen levels tends to decrease the ERα expression levels (Rogers *et al*, 2007).

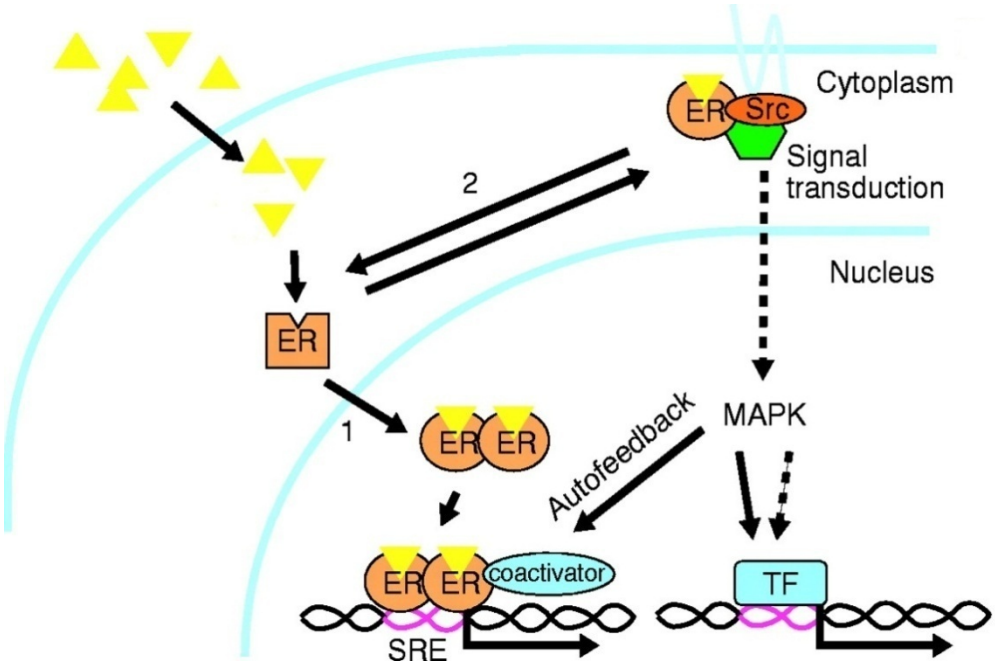


**Diagram 7: Genomic and non-genomic actions of the ERs.** Estrogenic ligands (triangles) activate ERs as a transcription factor (1) by inducing conformational changes that leads to its nuclear translocation, dimerization, and binding to specific steroid receptor response elements (SRE) in promoters of primary target genes. Activated ER recruits coactivators that are essential for assembly of a productive transcription complex at the promoter. Alternatively, a subpopulation of ER associates in an estrogen-dependent manner (2) with cytoplasmic- and/or cell membrane–signaling molecules (shown here is the tyrosine kinase Src). This extranuclear interaction promotes the Shc-Src-Raf–MAPK kinase (MEK)–MAP kinase phosphorylation cascade. Because MAPK can directly (solid arrows) or indirectly (dashed arrows) activate other transcription factors (TF), the MAP kinase pathway can potentially regulate distinct or complementary sets of genes from those regulated by nuclear ER pathways. Estrogen-activated MAPK can also enhance the direct transcriptional activity of ER by an auto-feedback loop through phosphorylation of ER or ER-associated co-activators. The green hexagon indicates an adaptor protein. Image adapted from *Mol Interv*., 3, 2003 (Edwards & Boonyaratanakornkit, 2003)

Estrogen is considered renoprotective. Chronic renal disease development in menopausal females is suggested to be due to loss of ovarian estrogen. It was shown in female Dahl salt-sensitive rats that ovariectomy increased renal diseases due to increased renal inflammation and the damage was reduced with E2 supplement (Maric *et al*, 2008). Decrease in ERα expression following ovariectomy contributed to renal damage and hypertension in female Dahl salt sensitive rats (Esqueda *et al*, 2007). Estrogen is reported to regulate extracellular matrix (ECM) kinetics in kidney by increasing matrix metalloproteinase synthesis and decreasing type I and IV collagen synthesis, thus protecting kidney from damage and fibrosis (Dixon & Maric, 2007, Mankhey *et al*, 2005, Mankhey *et al*, 2007, Maric & Sullivan, 2008, Neugarten *et al*, 2000, Potier *et al*, 2001, Potier *et al*, 2002, Silbiger *et al*, 1998).

## 2.6 Estrogen and Inflammation

The anti-inflammatory, as well as pro-inflammatory effects of estrogen, at various doses in different cell types and under different physiological conditions have been numerously demonstrated (Straub, 2007). Recent studies have shown the presence of estrogen receptors on the cells involved in the immune response, namely thymocytes, macrophages, and endothelial cells (Straub, 2007). Furthermore, cytokine production has also been shown to vary with changes in the circulating levels of estrogen in females during pregnancy and menopause (Diagram 8) (Straub, 2007). Physiological estrogen levels have been reported to have positive protective effects on the kidney tissue. For example, estrogen has been shown to inhibit the IL-6 production in Kupffer cells and thus reduce the risk of liver cancer in female mice (Naugler *et al*, 2007). In a study IL6

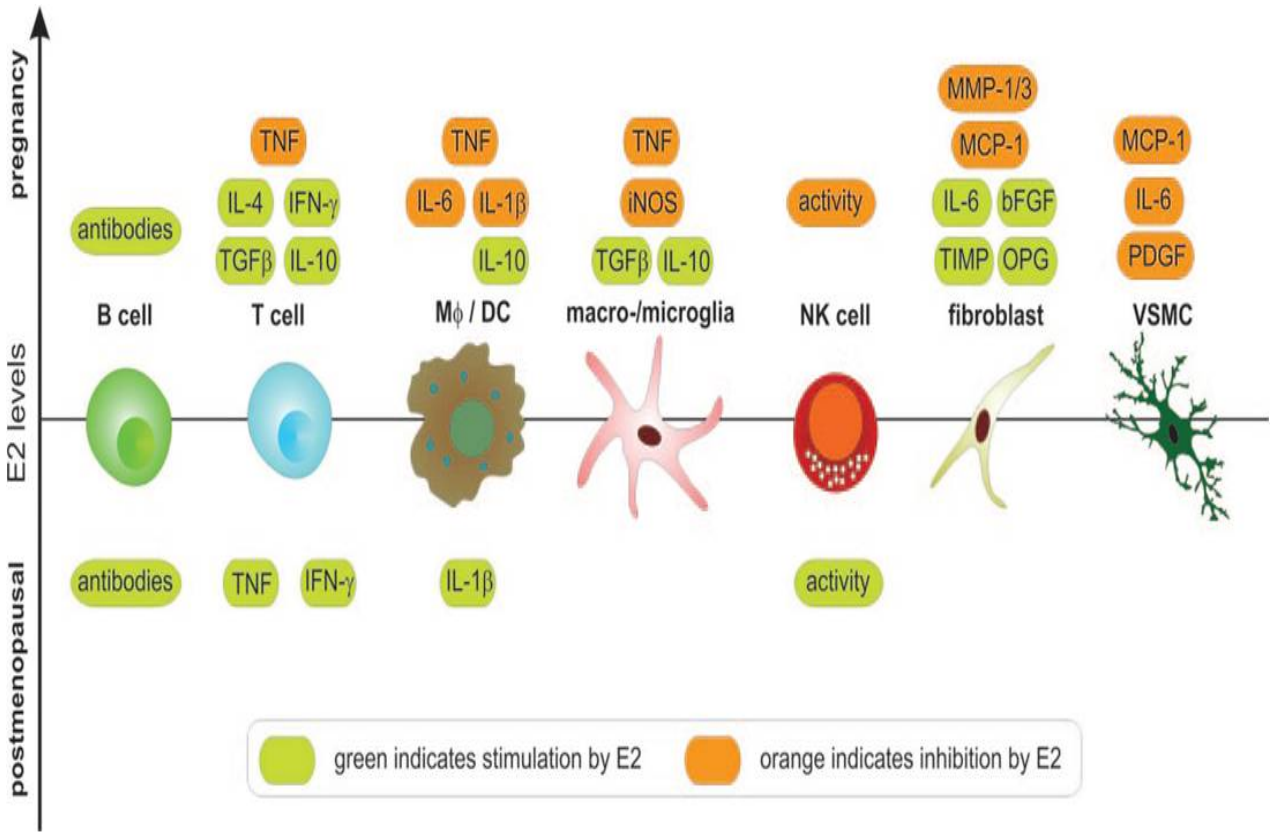


**Diagram 8: Dichotomy of the effects of estrogen on inflammatory pathway during pregnancy and menopause**. Influence of estrogen on several pro and anti inflammatory pathways at different estrogen doses as determined by various studies on different cell types. Depending on the dose of estrogens, factors in green boxes are stimulated while factors in orange boxes are inhibited by estrogens. Estrogen at doses found during pregnancy appears to be more anti-inflmmatory, while reduced estrogen levels during menopausal stages seems to promote pro-inflammatory conditions. DC, Dendritic cell; MØ, monocyte/macrophage; NK cell, natural killer cell; OPG, osteoprotegerin; PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell. Image adapted from *Endocr. Rev.*, 28, 2007 (Straub, 2007)

expression in human retinal pigment epithelial cells was found to be down-regulated by estrogen treatment following TLR activation (Paimela *et al*, 2007). ERα activation was also shown to inhibit the IL-1β gene induction in the mice livers (Evans *et al*, 2002). In addition, IFN γ production in the spleens was found to be increased by approximately 4-fold in ERα-deficient mice over wild type mice, suggesting a suppressing role for ERα in IFNγ production during chronic bacterial infection (Curran *et al*, 2004). Estrogen mediated regulation of endothelial nitric oxide synthase (NOS) expression is known to control renal inflammation (Maric *et al*, 2008, Satake *et al*, 2008). Estrogen treatment of endometrial epithelial cells stimulated with TLR3 ligands showed repression of chemokines and cytokine expression via ERα activation pathway (Lesmeister *et al*, 2005). TLRs expressed on renal epithelial cells and macrophages are activated by bacterial components to induce chemokines and cytokine secretion. Yao *et al* reported change in expression levels of TLRs in murine vaginal epithelium in different stages of estrous cycle (Yao *et al*, 2007). Moreover, men with chronic renal failure experience more rapid decline in renal function than women (Straub, 2007).

## 2.7 Estrogen and UTI

Incidence of UTI seems to vary with age and stages of menstrual cycle suggesting hormonal regulation of UTI susceptibility. Postmenopausal women have high prevalence of UTI and estrogen deficiency appears to be an important contributing factor (Altoparlak *et al*, 2004, Foxman *et al*, 2001, Franco, 2005, Pabich *et al*, 2003). Moreover, in canine studies, it has been reported that uterus infection in bitches occur during the first half of

the diestrous stage of estrous cycle, when the estrogen dose is the lowest (Sugiura *et al*, 2004). Estrogen treatment in postmenopausal women is reported to reduce the incidence of UTIs and extent of inflammation induced tissue damage following UPEC infection (Devillard *et al*, 2004, Heinemann & Reid, 2005, Maloney, 2002, Perrotta *et al*, 2008, Rozenberg *et al*, 2004, Stamm, 2007, Stern *et al*, 2004, Valiquette, 2001). Estrogen treatment in postmenopausal women was found to be protective against UTI by contributing towards restoration of the periurethral and vaginal microflora and reduction of the risk of developing vaginal atrophy (Altoparlak *et al*, 2004, Devillard *et al*, 2004, Franco, 2005, Perrotta *et al*, 2008, Raz & Stamm, 1993, Rozenberg *et al*, 2004, Stamm, 2007). However, the exact mechanism of estrogen mediated protection is not completely understood and appears to be more complex involving functions extending beyond restoration of commensal microflora and organ structure. Studies until now have explored the effects of estrogen on UTI susceptibility in postmenopausal women and have shown contradictory outcomes (Curran *et al*, 2007, Hu *et al*, 2004). In a study by Perrotta C. *et al*, it was found that estrogen treatment reduced the number of UTIs in postmenopausal women suffering with recurrent UTI. However the protection varied according to the type of E2 used and the duration of the treatment given (Perrotta *et al*, 2008). Increased susceptibility for UTI in an experimental mouse model following high dose of estrogen treatment was recently reported (Curran *et al*, 2007). The variation in estrogen doses used, the target organs studied and the age groups of the study populations may contribute to the conflicting results observed in different studies in this direction (Stamm, 2007). The effects of estrogen may vary as there is variation in circulating estrogen doses in pubertal, menstruating, menopausal, and postmenopausal female. In

the study by Curran *et al,* increased risk for UTI was found in the kidneys but not in the bladders suggesting differential effects on lower and upper urinary tract at that dose of estrogen used (Curran *et al,* 2007). Despite numerous studies, the exact mechanism of estrogen mediated immunomodulation during UTIs remains an enigma.

Physiological levels of estrogen have an important role to play in modulating the risk factors for urinary tract infection. Increased (pregnancy) as well as decreased levels of estrogen (menopause, hysterectomy, and ovariectomy) are considered as factors contributing to UTI susceptibility. In addition, long term hormone therapy with estrogen seems to have some non beneficial outcome in treatment groups (Ahmed *et al*, 2008, Franco, 2005, Hooton, 2000, Ribeiro *et al*, 2002, Sonnex, 1998). Therefore, it seems necessary to explore the effects of estrogen on UTI susceptibility and immunomodulation at the physiological doses of estrogen in female urinary tract. This will guide us to understand the mechanistic roles of estrogen in different anatomical parts of the female upper and lower urinary tract. This information will help in determining more judicious use of estrogen in treatment prescription for females with differing physiological conditions and also to look for therapeutic alternatives including estrogen related drugs for UTI treatment.

# CHAPTER III

# RESEARCH DESIGN AND METHODOLOGY

## 3.1 Experimental Models for UTI induction

### 3.1.1 Uropathogen for induction of experimental UTI

We have used Dr+ *E. coli*, strain-IH11128 (O75:K5: $H^-$) for inducing experimental UTI *in vivo* and *in vitro* in the present study. Pure culture of Dr+ *E. coli* was obtained from Dr Bogdan Nowicki (University of Medical Branch at Galveston, Texas) and maintained in our laboratory. *E. coli* strain-IH11128 is a clinical isolate identified from a girl diagnosed with acute pyelonephritis and it has been further characterized as a UPEC bearing Dr adhesin (Nowicki *et al*, 1987, Vaisanen-Rhen *et al*, 1984, Vaisanen-Rhen, 1984). Dr+ *E. coli* is associated with cystitis, recurrent UTI and pyelonephritis in humans (Goluszko *et al*, 1997, Nowicki *et al*, 2001, Servin, 2005, Vaisanen-Rhen *et al*, 1984, Vaisanen-Rhen, 1984). This *E. coli* strain has been previously used to develop experimental mouse model for ascending urinary tract infection using C3H/HeJ mice (Goluszko *et al*, 1997, Kaul *et al*, 1999, Nowicki *et al*, 2001).

### 3.1.2 *In vivo* murine models for induction of experimental UTI

Mice are preferred *in vivo* model for studying pathogenesis of lower and upper urinary tract infections due to the predominance of glycolipids that form the attachment receptors for majority of UPEC in both human, as well as mouse kidney (Hagberg *et al*, 1983a). We have used TLR4 mutant female C3H/HeJ mice as well as ERα gene knockout (ERα-/-) and ERα gene intact (ERα+/+) female B6.129 mice for inducing experimental UTI in our study.

#### A) Menopausal C3H/HeJ murine model for *in vivo* experimental UTI induction

OVX TLR4 mutant-C3H/HeJ mice were used for the development of an experimental menopausal UTI murine model to study the effects of estrogen on the development of ascending UTI infection. TLR4 expressed on renal tubular cells mediate direct inflammatory responses against bacterial products, especially LPS by activating secretion of cytokines and chemokines. Mutations in TLR4 are associated with endotoxin hyporesponsiveness resulting in impaired inflammatory response and thus increased risk towards gram negative infections (Chowdhury *et al*, 2006, Scherberich & Hartinger, 2007, Tsuboi *et al*, 2002, Wolfs *et al*, 2002). Moreover, increased UTI infections in children with TLR4 mutation has also been reported (Karoly *et al*, 2007) and reduction in TLR4 expression has been observed to be associated with asymptomatic bacteriuria in children (Ragnarsdottir *et al*, 2007). C3H/HeJ mice carry a point mutation in the *tlr4* gene that results in replacement of proline with histidine at position 712 in the cytoplasmic domain of TLR4 leading to defective signaling. This causes the LPS hyporesponsive phenotype and increased susceptibility to bacterial infection (Poltorak *et*

*al*, 1998). These mice show increased susceptibility for *E. coli* mediated urinary tract infection (Hagberg *et al*, 1983b, Hagberg *et al*, 1984) and have been successfully used for developing the experimental chronic pyelonephritis model with Dr+ *E. coli* (Goluszko *et al*, 1997) and other UPEC (Chassin *et al*, 2006). We have therefore used OVX C3H/HeJ mice as an *in vivo* surgically induced menopausal mouse model for induction of experimental UTI to investigate the effects of estrogen on bacterial colonization and infection associated inflammation in the urinary tract. We have confirmed the expression of ER subtypes and Dr+ *E. coli* colonization receptors DAF and type IV collagen in the kidney of C3H/HeJ mice at mRNA levels by quantitative SYBR green realtime PCR and at protein levels by immunohistochemistry.

**B) ERα-/- murine model for *in vivo* experimental UTI induction**

To discern the role of ERα in determining susceptibility to Dr+ *E. coli* colonization and infection associated inflammation in kidney, we have used female ERα-/- mice. The ERα-/- B6.129-Estra$^{tm1}$N10 mice (Jackson Laboratory) carry disrupted ERα gene (ESR1) and completely lack functional ERα protein, however ERβ protein remains functional in these mice (Lubahn *et al*, 1993). ERα+/+ B6.129 female mice (Jackson Laboratory) were used as experimental control. We detected the expression of Dr+ *E. coli* colonization receptors DAF and type IV collagen in the kidney of ERα-/- and ERα+/+ mice at mRNA levels by quantitative SYBR green real-time PCR

### 3.1.3 *In vitro* cell culture models for induction of experimental UTI

The most distal renal tubule segments, the medullary collecting ducts are the first to come in contact with ascending UPEC and thus are the primary site of bacterial adherence and early inflammatory responses against UPEC (Chassin *et al*, 2006, Vandewalle, 2008). Immortalized kidney cells have been observed to induce cytokine and chemokines expression following interaction with UPEC (Chassin *et al*, 2006, Chassin *et al*, 2007). We have used the following immortalized kidney inner medullary collecting duct (IMCD) cell lines of mouse and human origin for establishing *in vitro* UTI model for the present study : Mouse and human inner medullary collecting duct cells (mIMCD3 and hIMCD)

The kidney IMCD cell lines, mIMCD3 and hIMCD cell were generous gifts from Dr. Hari. Koul (University of Colorado at Denver and Health Science Center, Denver, CO). The mIMCD3 cell line (ATCC #CRL-2123™) was derived from the terminal one-third of the IMCD tubule obtained from the transgenic mouse carrying genes for the early region of SV40 [Tg(SV40E)bri/7]. This is a polarized epithelial cell line which retains many differentiated characteristics of the terminal IMCD (Rauchman *et al*, 1993). The hIMCD cell line was developed from normal papillary tissues dissected from the surgical waste of consenting patients undergoing renal surgery and was shown to retain expression of several epithelial cell markers and characteristics of IMCD cells (Khandrika *et al*, 2008). We established an *in vitro* experimental UTI model using mIMCD3 and hIMCD cells to study the invasion of pyelonephritic Dr+ *E. coli* strain in the kidney IMCD cells and to further investigate the molecular mechanisms underlying uropathogenesis and estrogen mediated protection during infection with Dr+ *E. coli*. In

the present study we have performed most of our *in vitro* experiments using mIMCD3 cells. We confirmed the expression ER subtypes and Dr+ *E. coli* colonization receptors DAF and type IV collagen in mIMCD3 and hIMCD at mRNA levels by quantitative SYBR green real-time RT-PCR using either SYBR green or TaqMan chemistry. We detected the protein expression of DAF in mIMCD3 and hIMCD by western blot. Surface expression of DAF on mIMCD3 cells was determined by flow cytometry. ER subtypes expression at protein level in mIMCD3 was detected by immunocytochemistry and western blot.

## 3.2 *In vivo* Methods

### 3.2.1 Animals and Animal care

A) **C3H/HeJ mice:** Six weeks old OVX (ovariectomy at 5 weeks), and Non-OVX C3H/HeJ mice were purchased from Jackson Laboratories. Following arrival, mice were kept in microisolater cages and were housed in a room with controlled temperature and a 12 hrs light-dark cycle in the animal facility of the Oklahoma State University, Center for Health Sciences. They had free access to filtered water and regular diet (Harlan Teklad Global diets #2018S). The animals were rested for a week following arrival and categorized into different groups as per their treatments and infection scheme (Table 1).

B) **ERKO mice:** Female ERα-/- B6.129-Estra$^{tm1}$N10 mice and ERα+/+ mice were obtained as gifts from Dr Susan Kovats, Oklahoma Medical Research Foundation,

OK. Mice were transported from the animal facility of OMRF to the animal facility of the Oklahoma State University, Center for Health Sciences in two batches at an interval of one month. The first batch included five 20-32 weeks old ERα-/- mice and ten 20-32 weeks old ERα+/+ mice. The second batch included five 8-10 weeks old ERα-/- mice and five 8-10 weeks old ERα+/+ mice. Following arrival, mice were kept in microisolater cages and were housed in a room with controlled temperature and a 12 hrs light-dark cycle, in the animal facility of the Oklahoma State University, Center for Health Sciences. They had free access to filtered water and regular diet (Batch #1: Picolab mouse lab diet 20 # 5058 and Batch #2: Harlan Teklad Global diets #20185). The animals were rested for a week following arrival and categorized into different groups as per their infection scheme (Table 2).

Following housing the animals, the cage's soiled bedding were routinely changed. The microisolater cages with infected bedding containing excretion of animals were treated as contaminated and were sprayed with 10% bleach and autoclaved in biohazard bags before disposal. Cages were washed, acid rinsed and thoroughly decontaminated after beddings were removed. All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals approved by Oklahoma State University, Center for Health Sciences. All animals were used for experiments within two weeks of delivery.

### 3.2.2 Animal Treatment

OVX C3H/HeJ mice were treated with a physiological dose of E2 (17, β-estradiol Sigma, E 4389) or vehicle (sterile PBS+corn oil). Non-OVX mice were treated with ICI 182, 780 (Tocris 1047) or vehicle (sterile PBS+corn oil). E2 dissolved in PBS was mixed with corn oil (1:2 vol/vol) and 100 µL of this mixture was administered subcutaneously into each mouse before induction of experimental UTI. Each mouse received either 6 µg of E2 /kg/day in corn oil or received corn oil with PBS per day, for five consecutive days prior to infection. ER antagonist ICI 182, 780 dissolved in ethanol and diluted in PBS was administered along with corn oil at the rate of 5 mg/kg/day to Non-OVX mice in the a similar way as described for E2. Treatment with ICI 182, 780 was given for 5 days prior to UTI induction and continued for 6 days post-UTI induction. The dose of E2 treatment (for treatment at physiological levels) and the dose for ICI 182, 780 used in this study have been described previously (Elloso *et al*, 2005, Sawada *et al*, 2000)

### 3.2.3 Culture of Dr+ *E. coli* and Hemmaglutination of red blood cells

Dr+ *E. coli* was revived from frozen stock and was grown on Luria Bertani agar (LB) plates (Fisher Scientific) overnight to maintain the culture. Pure cultures of Dr+ *E. coli* strain were routinely tested for hemagglutination using a 30% suspension (vol /vol) of human O erythrocytes in phosphate-buffer saline (PBS).

### 3.2.4 Dr+ *E. coli* inoculum preparation for induction of experimental UTI

Bacterial suspension prepared with pure culture of Dr+ *E. coli* was used to induce experimental UTI in mice as described before (Kaul *et al*, 1999). Dr+ *E. coli* was

cultured on LB plates at 37°C overnight. Isolated colonies of Dr+ *E. coli* from overnight bacterial culture were used to prepare bacterial suspension in sterile PBS. Bacterial suspension with an optical density of 1 at 600nm (corresponding to approximately $10^8$ bacterial cells) was used as inoculum for experimental UTI induction. Bacterial counts in bacterial suspension were determined by plating 10-fold serial dilutions of bacterial suspension on LB plates and counting the viable colonies following 18 hrs of incubation at 37º C. The hemagglutination property of the Dr+ *E. coli* pure culture was tested before inoculum preparation.

### 3.2.5 Experimental UTI induction in mice

Induction of experimental UTI in mice was performed by bladder catheterization. The mice were anesthetized by isoflurane inhalation and 50µl of bacterial inoculum was instilled slowly into the bladder through a soft catheter with an outer diameter of 0.30 mm (Norton Performance Plastics Soft Plastics, Akron, Ohio) adapted to a needle on a tuberculin syringe (0.4 by 20 mm). After injection, the catheter was immediately withdrawn and no further manipulations were performed. Controls were similarly injected with 50 µl of sterile PBS. Following infection, mice were retumed to their cages and monitored daily for any adverse symptoms or changes in body weight until euthenized. Mice were euthanized by $CO_2$ inhalation followed by cervical dislocation. Kidneys and bladders were aseptically harvested and snap frozen. Summary of the infection scheme is represented in Table 1 for C3H/HeJ mice and Table 2 for ERα +/+ and ERα-/- mice. The *in vivo* research design for C3H/HeJ mice is pictorially represented in Diagram 9.

**Table 1: Treatment groups for OVX and Non-OVX C3H/HeJ mice**

| | Number of mice | | | | |
|---|---|---|---|---|---|
| | **Infected (Dr+ *E. coli*)** | | **Uninfected** | | **Total** |
| **Days of infection →** | **2 days** | **6 days** | **2 days** | **6 days** | |
| **Treatments ↓** | | | | | |
| **OVX C3H/HeJ mice** | | | | | |
| E2 (6 μg/kg mouse /day in PBS + corn oil)* | 6 | 6 | 4 | 4 | **20** |
| Vehicle **(**PBS + corn oil**)** | 6 | 6 | 4 | 4 | **20** |
| **Non-OVX C3H/HeJ mice** | **3 days** | **7 days** | **3 days** | **7days** | |
| ICI 182,780 (5 mg/kg mouse/day in ethanol and PBS + corn oil)* | 5 | 5 | 0 | 2** | **12** |
| Vehicle (ethanol and PBS + corn oil) | 5 | 5 | 0 | 2 | **12** |
| **Total** | **22** | **22** | **8** | **12** | **64** |

*E2 treatments were continued for 5 consecutive days pre-UTI induction. ICI 182, 780 treatment was given for 5 consecutive days pre-UTI induction and then continued for 6 consecutive days post-UTI induction; ** 1 mouse died during the treatment

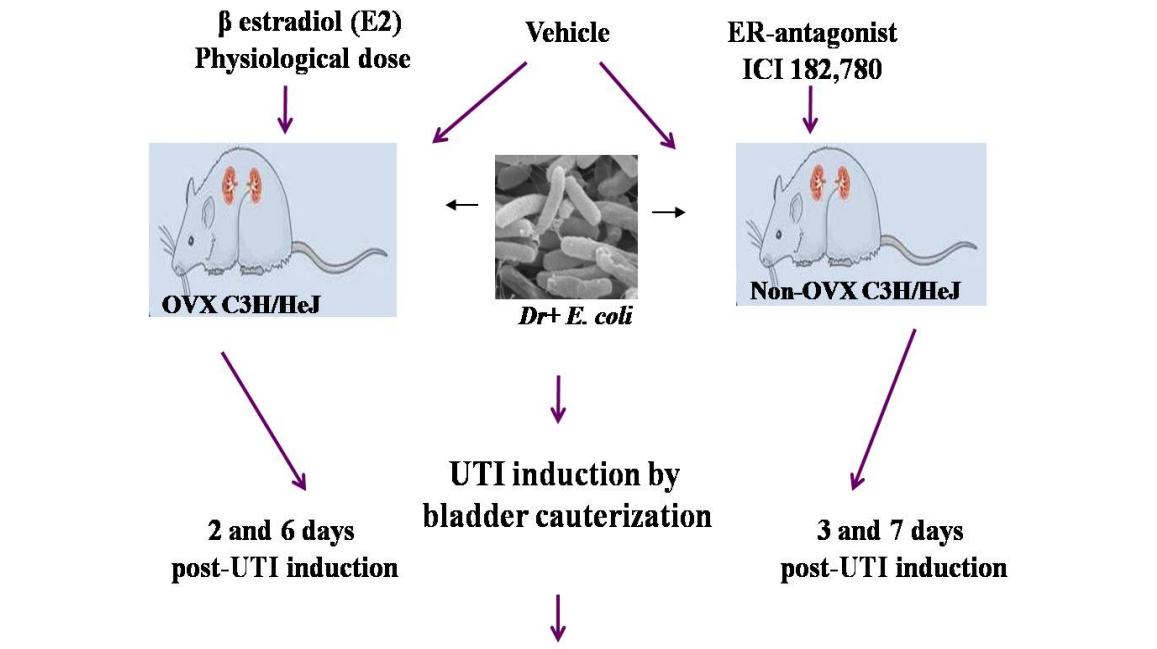


**Diagram 9: *In vivo* research design using C3H/HeJ mice.** Diagramatic representation of research design for studying bacterial colonization, expression of colonization receptors and inflammatory responses following Dr+ *E. coli* infection using the *in vivo* C3H/HeJ murine model

**Table 2: Infection groups for ERα-/- and ERα+/+ mice**

| | **Number of mice** | | | | **Total** |
|---|---|---|---|---|---|
| | **Infected (Dr+ *E. coli*)** | | **Uninfected** | | |
| **Days of Infection** | **3 days** | **7 days** | **3 days** | **7 days** | |
| **Experiment I** | | | | | |
| **ERα-/-** | 2 | 3 | 0 | 0 | **5** |
| **ERα+/+** | 4 | 4 | 0 | 2 | **10** |
| **Experiment II** | | | | | |
| **ERα-/-** | 0 | 5 | 0 | 0 | **5** |
| **ERα+/+** | 0 | 5 | 0 | 0 | **5** |
| **Total** | **6** | **17** | **0** | **2** | **25** |

### 3.2.6 Tissue homogenate culture

The aseptically removed kidney and bladder tissues were weighed and homogenized in sterile PBS using an Omini Tip homogenizer. The number of viable Dr+ *E. coli* in the tissues was determined by plating tissue homogenates on LB plates in duplicates. The plates were incubated overnight at 37ºC and the numbers of bacterial colonies obtained were counted manually on a colony counter grid. The bacterial counts represented viable bacteria present in the bladder and kidney homogenates. Counts of bacterial colonies obtained were expressed as the number of CFU per gram tissue weight. Summary of the infection scheme is represented in Table 1 for C3H/HeJ mice and Table 2 for ERα +/+ and ERAα-/- mice.

### 3.2.7 Serum Preparation

Blood samples were collected from mice by tail bleeding and centrifuged for 20 minutes at 16000 rpm at 4°C. Clear serum was collected from each sample transferred to fresh tubes and stored at80°C.

### 3.2.8 Measurement of serum E2 levels

Serum E2 dose was measured in each group using a competitive EIA kit (Oxford Biomedical Research # EA70).

### 3.2.9 Measurement of serum TNFα levels

Serum TNFα dose was measured in ERα-/- and ERα+/+ mice using mouse TNFα ELISA kit ( eBiosciences # 88-7324-22)

## 3.3 *In vitro* Methods

### 3.3.1 Cell culture

The mIMCD3 cells were cultured and maintained in Dulbecco's modified Eagle's Medium (DMEM-F12), (Invitrogen/Gibco) and hIMCD cells were cultured and maintained in DMEM (Invitrogen/Gibco). The cell culture medium was supplemented with 5% FBS (Atlanta Biologicals) and antibiotics: 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were plated in T-25 or T-75 flasks and cultured in a humidified atmosphere (95%) containing 5% $CO_2$ at 37°C Cell cultures were maintained by subculturing and were passaged at a ratio of 1:4 once they became 80-90% confluent (every 3-4 days).

### 3.3.2 Drug treatment

For all the drug treatment experiments, mIMCD3 cells were plated at a dose of 0.5 million cells per well in 12 well plates and cultured in a medium supplemented with antibiotics and 5% heat-inactivated fetal bovine serum depleted of steroids by dextran-charcoal treatment (charcoal stripped serum : VWR). The cells were allowed to grow and attach overnight (18-24 hrs) followed by treatement with different drugs (Table 3). For drug treatments, the cell monolayers grown in 12 well plates were washed twice with PBS gently and serum free culture medium containing various doses of drugs was added to each well. For untreated control cells, no drug was added to the culture medium. The

stock solutions of the compounds: E2 (Sigma-Aldrich), ICI 182, 780, PPT, DPN, MPP, R, R-THC, Genestein or LY294002 (Tocris Bioscience) were diluted up to the required working doses in serum free media. The cells were incubated for desired period of time with the drugs prior to infection with Dr+ *E. coli.* For mIMCD3 cells, the doses used and the duration of treatment for each drug is described in Table 3. The hIMCD cells were treated with E2 at 1, 10 and 50 nM doses for 24 hrs prior to infection. The *in vivo* research design for mIMCD3 cells is pictorially represented in Diagram 9.

### 3.3.3 Gentamicin Protection Assay

Following infection with Dr+ *E. coli* for 2 hrs, non adherent bacterial cells were aspirated, and loosely adherent bacterial cells were removed by washing the infected cells monolayers with PBS atleast 2-3 times. The cell monolayers were then incubated with 50µg/ml gentamicin for 90 minutes to kill the extracellular adhering bacteria. Gentamicin was aspirated and cell monolayers were washed thrice with PBS to remove the residual gentamicin. The cells were then permeabilized with lysis buffer (1% Triton X-100 in PBS), following which the cell lysates were spread on LB plates in duplicates and incubated at 37°C overnight. Bacterial colonies that appeared after 18 hrs of incubation were counted and represented as colony forming unit (CFU). The percentage of bacteria invasion in drug treated cells was determined relative to the invasion in untreated control cells (Duncan *et al*, 2004). Invasion in untreated control cells was considered 100%.

**Table 3: Drug treatment in mIMCD3 cells**

| Drug | Drug Action | Doses used for treatment | Duration of treatment |
|---|---|---|---|
| 17, β-Estradiol (E2) | ER-agonist | 1 nM, 10 nM, 50 nM, and 100 nM | 24hrs |
| ICI 182, 780 | ER-antagonist | 0.1 µM, 1 µM, and 10 µM | 24hrs |
| PPT | ERα selective agonist | 0.01 µM, 0.1 µM, and1 µM | 24hrs |
| DPN | ERβ selective agonist | 0.01 µM, 0.1 µM, and1 µM | 24hrs |
| MPP | ERα selective antagonist | 0.01 µM, 0.1 µM, and1 µM | 24hrs |
| R,R-THC | ERβ selective antagonist | 0.1 µM, 1 µM, and 10 µM | 24hrs |
| Genistein | Tyrosine kinase inhibitor | 0.1 µM, 1 µM, and 10 µM | 24hrs |
| LY294002 | PI3K inhibitor | 10 µM, 50 µM, and 100 µM | 15 min |
| Co-treatment of cells with 10 nM E2 and various doses of ICI 182, 780, MPP or R,R-THC was performed for 24hrs. | | | |

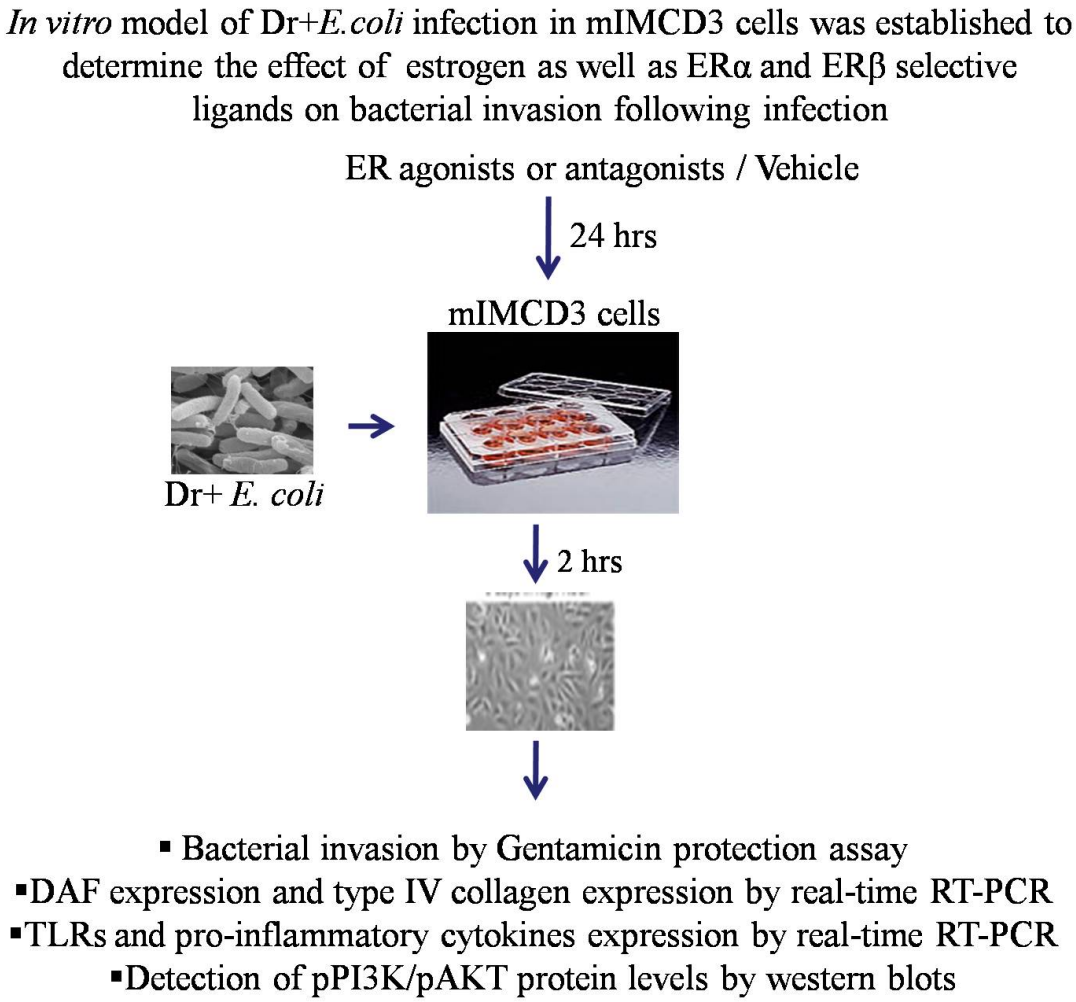


**Diagram 10: *In vitro* research design using mIMCD3 cells.** Diagramatic representation of research design for studying the effects of estrogen and ER subtype specific ligands on bacterial invasion, expression of colonization receptors, and inflammatory responses, following Dr+ *E. coli* infection *in vitro*, using mIMCD3 cell culture model.

For enumeration of total infection, (bacteria adhered and internalized), following 2 hrs of infection, the cell monolayers were washed with PBS thrice after aspirating the bacterial suspension. The cells were then lysed with the lysis buffer and the lysates were cultured on LB plates at 37°C for 18 hrs. The bacterial colonies that appeared were counted and represented as CFU.

### 3.3.4 MTT test for cell toxicity

Cell viability in response to treatment with different drugs was determined colorimetrically by measuring the reduction of tretrazolium salt MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromid) to insoluble formazan by mitochondrial dehydrogenase. Cells (mIMCD3 and hIMCD) were cultured in 96 well plates at a dose of 50,000 cells per well in 200 µl of culture media. The confluently growing cells were then incubated with different drugs (Table 3) for desired period of time (Table 3). Following incubation 10mg/ml MTT (Sigma) solution was added to the media in each well and incubated for 2hrs at 37°C. The media was the aspirated and 100 µl of dimethyl sulphoxide (DMSO) was added to each well to dissolve the developed formazan. Absorbance was measured at 570 nm. The absorbance values from wells containing drug treated cells were compared to the values from untreated control cells to determine the viability of cells in each treatment group.

## 3.4 Quantitative analysis of mRNA expression

### 3.4.1 RNA isolation and cDNA synthesis

Total RNA was isolated from kidney tissues and mIMCD cells with Trizol reagent (Invitrogen) using manufacturer's instructions. Inner medullary and renal pelvis regions were dissected out from the kidney tissues and were used for RNA isolation. RNA samples were quantitated on ND-1000 (NanoDrop Technologies) and their integrity was determined by denaturing agarose gel electrophoresis. RNA samples showing protein and chemical contamination as determined by 280/260 and 230/260 ratio were purified using RNeasy columns (Qiagen), using the manufacturer's instructions. Total RNA from each sample was given DNase treatment (Ambion) and then reverse-transcribed to cDNA, using High capacity cDNA synthesis kit (Applied Biosystems), following the kit's instructions.

### 3.4.2 Quantitative real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

Quantitative real-time RT-PCR was performed using TaqMan or SYBR green chemistry on an ABI StepOne Real-Time PCR System (Applied Biosystems). PCR Reactions were performed using cDNA as template (approximately 10-100 ng), target gene and endogenous control specific primers and reaction master mix. SYBR green PCR primers were purchased from realtimeprimers.com (Table 4), and TaqMan primers were purchased from Applied Biosystem (Table 5). Power SYBR green master mix (Applied Biosystems) or GoTaq qPCR master mix (Promega) was used for SYBR green

**Table 4: Primer pairs for SYBR green real-time RT-PCR (realtimeprimers.com).**

| Target Gene Name | Forward and Reverse primer sequence(5'-3') | Primer Tm | Amplicon size | Gene Accession | Regognition region on genome |
|---|---|---|---|---|---|
| **ESR1(ERα)** | Fp: TTCTCCCTTTGCTACGTCAC<br>Rp: TCGCTTTGTCAACGACTTC | 58°C | 212bp | NM_007956 | 2208-2419 |
| **ESR2 (ERβ)** | Fp: GGTCATCAAATCGACCTTT<br>Rp:GGAACAAGGTCACATCCAAG | 58°C | 185bp | NM_207707 | 2413-2597 |
| **Collagen Type IVa1 Primerset#3** | Fp: CCACTGTCCAGTGTTTCCAC<br>Rp:CCACACAGGGCATAAGTTTG | 59°C | 132bp | NM_009931 | 5654-5785 |
| **DAF/CD55 Primerset#3** | Fp: AGCAAAGTGGCATACTCGTG<br>Rp: CTGGGAGTGGAGGTTGTTTT | 59°C | 247bp | NM_010016 | 246-492 |
| **TLR2** | Fp: GACGACTGTACCCTCAATGG<br>Rp: TAAATGCTGGGAGAACGAG | 58°C | 226bp | NM_011905 | 1205-1430 |
| **TLR4 Primerset#2** | FP: AAGGTTGAGAAGTCCCTGCT<br>Rp: TTGTTCTCCTCAGGTCCAAG | 58°C | 207bp | NM-021297 | 2232-2538 |
| **TNFα** | Fp: CCCACTCTGACCCCTTTACT<br>Rp: TTTGAGTCCTTGATGGTGGT | 58°C | 201bp | NM_013693 | 899-1099 |
| **Mip 2** | Fp: TCATGGAAGGAGTGTGCAT<br>Rp: CACGAAAAGGCATGACAAA | 59°C | 216bp | NM_009140 | 803-1018 |
| **IL6** | Fp: GGAGAGGAGACTTCACAGA<br>Rp: CAGTTTGGTAGCATCCATC | 58°C | 218bp | NM-_031168 | 122-339 |
| **Mouse endogenous control for relative expression studies** | | | | | |
| **ACTB** | Fp: AGAGCTATGAGCTGCCTGA<br>Rp: ACGGATGTCAACGTCACAC | 58°C | 160bp | NM_007393.1 | 792-951 |
| **B2M** | Fp:GGCCTGTATGCTATCCATAA<br>Rp:GAAAGACCAGTCCTTGCTGA | 58°C | 198bp | NM_009735.2 | 103-300 |
| **GAPDH** | Fp:CTGGAGAAACCTGCCAAGTA<br>Rp:TGTTGCTGTAGCCGTATTCA | 58°C | 223bp | BC083080.1 | 796-1018 |
| **GUSB** | Fp:GACTCAAAGGTGTCCCCAGA<br>Rp: CTTCACTCCAGCCTCTCACC | 58°C | 227bp | NM_010368.1 | 1981-2254 |
| **HPRT1** | Fp: GCTGACCTGCTGGATTACAT<br>Rp:TTGGGGCTGTACTGCTTAAC | 58°C | 242bp | NM_013556.1 | 313-554 |
| **PGK** | Fp:GCAGATTGTTTGGAATGGTC<br>Rp:TGCTCACATGGCTGACTTTA | 58°C | 185bp | NM_008828.2 | 1123-1307 |
| **PPIA** | Fp:AGCTCTGAGCACTGGAGAGA<br>Rp:GCCAGGACCTGTATGCTTTA | 58°C | 178bp | AK028210 | 155-332 |
| **RPL13A** | Fp:ATGACAAGAAAAAGCGGATG<br>Rp:CTTTTCTGCCTGTTTCCGTA | 58°C | 215bp | Bbc086896 | 358-572 |

**Table 5: Primer pairs for TaqMan real-time RT-PCR (Applied Biosystems).**

| Target Gene Name | Assay ID | Amplicon length | Exon | Interrogated sequence |
|---|---|---|---|---|
| **Mouse specific primers** | | | | |
| **ESR1(ERα)** | **Mm00433149_m1** | **56** | **4&5** | **NM_007956.4** |
| **ESR2 (ERβ)** | **Mm00599819_m1** | **71** | **6&7** | **NM_010157.3** |
| **PPIA** | **Mm02342429_g1** | **112** | **3&4** | **NM_008907.1** |
| **Human specific primers** | | | | |
| **ESR1(ERα)** | **Hs01046816_m1** | **65** | **6&7** | **NM_001122740** |
| **ESR2 (ERβ)** | **Hs00230957_m1** | **63** | **4&5** | **NM_001040275** |
| **PPIA** | **Hs01893911_s1** | **90** | **1&1** | **AK123006.1** |

PCR reactions. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for TaqMan PCR reactions. Standard TaqMan or SYBR green protocols were followed for PCR amplification on ABI StepOne Real-Time PCR System. Melt curve analysis was performed for all the SYBR green real-time PCR reactions. Reaction products were checked on 2% agarose gel to confirm the correct amplicon size generated following PCR amplification of each target. We evaluated expression of several target genes (Table 4) as candidates for endogenous control for relative expression analysis. Expression of PPIA (peptidylprolyl isomerase A also known as cyclophilin A) was determined to be the appropriate endogenous control for our gene expression studies *in vitro* as well as *in vivo.* Primer sequence information for target genes and endogenous controls is presented in Table 4. RNA standard curve was prepared for all the targets to ensure 90-100% RT efficiency of the reaction for each target. A validation experiment was also conducted for each set of primers using serial-fold dilutions of cDNA in comparison with the endogenous control to ensure that amplification efficiency falls between 95-100%. All PCR experiments were performed with hot start and were run in duplicates. Relative differences in gene expression between groups were expressed using cycle time values ($C_T$). The expression of target genes was normalized to the expression of PPIA (endogenous control) and delta $C_T$ was calculated where, $\Delta C_T = C_T$ target-$C_T$ endogenous control. Relative expression using comparative $\Delta C_T$ or $\Delta\Delta C_T$ ($\Delta\Delta C_{T=}$ $\Delta$CT of target of treatment group- $\Delta$CT of target of the calibrator group) method was determined and relative gene expression (RQ) was calculated as $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$. The results were expressed as relative expression of target genes in the kidney tissues for each group of mice and for each treatment group of mIMCD3 cells.

## 3.5 Analysis of protein expression

### 3.5.1 Protein isolation

Cells were washed with PBS and incubated on ice for 20 minutes in RIPA buffer (cell lysis buffer- Cell Signaling,) supplemented with 2mM phenylmethanesulfonyl fluoride (PMSF). Cells were then scraped and lysed by gently pipetting up and down. The lysates were briefly sonicated for 5 seconds followed by centrifugation at 16,000 x g for 30 minutes at 4ºC. The supernatants were collected and stored at -80ºC until analyzed. Protein dose of the extracts was determined with Pierce BCA (bicinchoninic acid) protein reagent kit (Pierce) using manufacturer's instruction.

### 3.5.2 Western Blotting

Twenty to 40 μg of cell lysate protein was subjected to gel electrophoresis on commercially available 4-12% pre-casted Bis–Tris Gel (Invitrogen). The separated proteins were transferred electrophoretically onto a 0.2 μM nitrocellulose membrane. The membranes were blocked in blocking buffer (5% non fat dry milk in Tris Buffer Saline with 0.1% Tween 20-TBST) for 2 hrs and incubated overnight at 4°C with appropriate dilutions of antigen specific primary antibodies (Table 6). Following washing with TBST, primary antibody binding was detected by incubating the membranes with appropriate dilutions of alkaline phosphatase (AP) labeled species specific secondary antibody (Sigma) for 2 hrs at room temperature. The resultant complex was detected by chemiluminescence detection system (Lumi-Phos TM, WB, and Pierce). The membranes were stripped and re-probed for detecting more than one antigen on the same membrane. Equivalent protein loading and transfer efficiency was verified by staining for β-actin.

**Table 6: List of primary and secondary antibodies**

| Antigen | Antibody Catalog # | Isotype | Source | Amount of Antibody | Application |
|---|---|---|---|---|---|
| Mouse ERα | (Mc-20), #sc-542 | Rabbit polyclonal | Santa Cruz | 1:1000 (WB) 1: 50 (ICC) | Western blot, Immunocytochemistry |
| Mouse ERβ | (PPG5/10)# M2792 | Mouse | Dako | 1:50 | Western blot, Immunocytochemistry |
| Mouse ERβ | (H-150) #Sc-8974 | Rabbit polyclonal | Santa Cruz | 1:1000 | Western blot |
| Mouse DAF/CD55 | *(2C6)- | Rat IgG2a | *Dr. Claire Harris | 10μg/ml | Western blot, |
| Human DAF/CD55 | (278803) #MAB2009 | Mouse IgG2b | R&D Systems | 2μg/ml | Western blot, |
| Mouse DAF/CD55 | 558037 (RIKO-5) | Hamster monoclon-al IgGλ3 | BD Pharmingen | 1μg/ml | Flow cytometry |
| Isotype control | 553980 | Hamster IgGλ3 | BD Pharmingen | 1μg/ml | Flow cytometry |
| Mouse Phosphorylated PI3-K 5(T458) | 4228 | Rabbit polyclonal | Cell signaling | 1:1000 | Western blot, |
| Mouse Total PI3-K | 4292 | Rabbit polyclonal | Cell Signaling | 1:1000 | Western blot, |
| Mouse Total Akt | 9272 | Rabbit polyclonal | Cell Signaling | 1:1000 | Western blot, |
| Mouse Phosphorylated Akt (S473) | 9271 | Mouse IgG2b | Cell Signaling | 1:1000 | Western blot, |
| β-Actin | A5060 | Rabbit polyclonal | Sigma | 1:2000 | Western blot, |
| Anti-Rabbit | A3687 | IgG | Sigma | 1:30,000 | Western blot |
| Anti-Rat | A3438 | IgG | Sigma | 1:30,000 | Western blot |
| Anti-Mouse | A3562 | IgG | Sigma | 1:30,000 | Western blot |
| WB:Western blot; ICC Immunocytochemistry ; * 2C6 an anti Mouse DAF antibody was a generous gift from Dr Claire Harris at Cardiff University School of Medicine, UK. | | | | | |

Images were digitally captured using Alpha Innotech Instrumentation (Alpha Innotech Corp). Quantitative analysis was performed using Image J software program (NIH Image, Scion Corp, Frederick MD).

### 3.5.3 Immunocytochemistry

Cells were plated at a dose of $1x10^5$ cells per well in 8-well chambered slides (BD Falcon Culture Slides, BD Biosciences;VWR) and cultured in DMEM F-12 medium supplemented with 5% fetal bovine serum and antibiotics. For staining, cell monolayer were washed gently with ice cold PBS and then fixed with methanol at 20ºC for 10 min (methanol fixation also causes delipidization and thus renders permeability to the cells). To block the endogenous peroxidase activity 1 drop of peroxidase block (Dako) was applied to each chamber such that the cells are completely covered and incubated for 6 min at room temperature (RT). The cells were washed twice with PBS. Excess buffer from and around the chambers was gently wiped off without touching the cells. For ERβ staining, Avidin /Biotin block was applied following peroxidase block. Cells were then incubated with PBS containing 3% BSA for 15 min for nonspecific antigen blocking and washed twice with PBS. Subsequently, the cells were incubated at 4°C overnight in a humidified chamber with ERα or ERβ specific antibody (Table 6). For negative control staining, cells were incubated with PBS containing 3% BSA and 0.1% Nonidet P-40 (buffer used for antibody dilution). Cells were then washed and incubated with peroxidase–labeled polymer conjugate to specific secondary antibody (Dako Envision system kit) for 30 min at room temperature. The cells were then washed and coveredwith DAB chromogen (3,3' Diaminobenzidine) substrate and incubated for 10 min at room

temperature. Following washing, cells were counterstained with hematoxylin and mounted with Super Mount mounting medium (Biogenex).

### 3.5.4 Flow cytometry

Cells growing in T-25 flasks were dissociated by incubating with 3 ml of cell dissociation buffer (Sigma) at 37º C for up to 20 minutes. Cells were removed by pipetting up and down gently. Cell suspension so obtained was centrifuged at 1100 rpm for 5 minutes at 4ºC and the cell pellet was suspended in cell suspension/wash buffer (PBS and 5% serum). Cells were then incubated with fluorescent labeled (R-phycoerythrin, also called PE) hamster anti-mouse DAF monoclonal antibody (RIKO 5) or an isotype-matched non-relevant control monoclonal antibody (Table 6) at a dose of 1µg per 1 X $10^6$ cells on ice for 60 min. Following staining, cells were analyzed in the Accuri C6 flow cytometer (Accuri Cytometers).

## 3.6 Statistical analysis

GraphPad Prism version 5 (Graph Pad Software Inc, San Deigo, CA) was used for statistical analysis. Student t-test or non parametric Mann Whitney U-test was performed for comparing two experimental groups. Data analysis for more than two experimental groups was conducted using either One-way or Two-way ANOVA followed by Tukey's, Bonferroni or Dunnett post hoc tests for multiple comparisons. Differences at $P< 0.05$ were considered significant (The character * signifies $P<0.05$, ** signifies $P<0.005$ and *** signifies $P<0.0005$).

# CHAPTER IV

# ESTROGEN MODULATES Dr+*ESCHERICHIA COLI* COLONIZATION IN THE THE MOUSE URINARY TRACT VIA ESTROGEN RECEPTOR ACTIVATION

## 4.1 Introduction

Clinical and animal studies have indicated that changes in the circulating ovarian hormone levels influence UTI susceptibility in females (Franco, 2005, Hooton, 2000, Ribeiro *et al*, 2002, Sonnex, 1998). Increased ovarian hormone levels during pregnancy and decreased ovarian hormone levels during menopause lead to increased incidence of UTI in women. In premenopausal women, adherence of *E. coli* to the uroepithelium is reported to vary through the menstrual cycle, and use of oral contraceptives also contributes significantly to an increase in bacterial adherence on the uroepithelium (Schaeffer *et al*, 1979, Sharma *et al*, 1987, Sonnex, 1998). The incidence of UTI in postmenopausal women is reported to be high; 20% incidence of recurrent UTI has been reported in community dwelling women, and over a 50% incidence has been reported in institutionalized women (Rozenberg *et al*, 2004). UTIs in postmenopausal women are mostly severe and are associated with a high rate of impaired kidney function, morbidity, and mortality (Brown *et al*, 2005, Dulawa, 2004, Molander *et al*, 2000).

Estrogen deficiency has been implicated as one of the contributing factors to postmenopausal UTIs, and supplementation of estrogen at low doses attenuate the progression of UTI in these women (Raz, 2001a, Stamm & Raz, 1999). However, the therapeutic benefits of estrogen replacement in postmenopausal women are not completely understood. Despite the beneficial effects observed, estrogen replacement therapy in postmenopausal women is associated with unwanted side effects. In addition, there are conflicting reports on the effects of route, duration, and dose of the estrogen administration during the therapy (Hextall, 2000, Raz, 2001a). Thus, it is important to understand the actions of estrogen in the female urinary tract and the possible molecular mechanisms associated with estrogen related etiology of UTI pathogenesis. This information will help in determining and improving effective treatment strategies for estrogen replacement therapy in estrogen deficient women.

Uropathogenic Dr+ *E. coli* colonize the host uroepithelium by attaching to hormonally regulated DAF and type IV collagen (Goluszko *et al*, 1997, Kaul *et al*, 1995, Kaul *et al*, 1996, Nowicki *et al*, 2001, Selvarangan *et al*, 2000, Selvarangan *et al*, 2004, Servin, 2005, Virkola *et al*, 1988). Folllowing 21 days post-experimental-UTI induction in the C3H/HeJ mice, we found increased Dr+ *E. coli* colonization in the kidneys of estrogen-deficient OVX mice compared to that in the kidneys of estrogen-sufficient Non-OVX mice and OVX mice treated with a physiological dose of E2. The present study was conducted to determine the protective role of estrogen on Dr+ *E. coli* colonization in the lower (bladders) as well as upper (kidneys) urinary tracts of C3H/HeJ mice at earlier time points during infection. Bacterial colonization in bladders and kidneys of OVX

mice and Non-OVX mice was observed and compared at 2 and 6 days post-UTI induction. We also tested the effects of E2 pretreatment at a physiological dose on the bacterial colonization in the OVX mice at these time points. To further determine the involvement of ERs in estrogen mediated modulation of Dr+ *E. coli* uropathogenesis, Dr+ *E. coli* colonization was observed in the ER-antagonist(ICI 182, 780)-treated Non-OVX mice, at 3 and 7 days post-infection. Since we have found (Figure 4) and other studies have also reported the predominance of ERα in the mouse kidney (Carley *et al*, 2003, Jelinsky *et al*, 2003, Sharma & Thakur, 2004), we employed ERα-/- and ERα+/+ female B6.129 mice to investigated the role of ERα in estrogen-mediated modulation of Dr+ *E. coli* colonization in the the mice urinary tractss.

We found that compared to the estrogen-sufficient Non-OVX mice, the estrogen-deficient OVX mice and the ER-antagonized Non-OVX mice show increased susceptibility to Dr+ *E. coli* colonization in the bladders, as well as in the kidneys, at earlier time points following infection. Furthermore, E2 replacement at a physiological dose led to a significant reduction in the bacterial colonization in the the mice urinary tracts. We further observed that compared to the ERα+/+ mice, the ERα-/- mice have increased susceptibility to Dr+ *E. coli* colonization in the upper, as well as in the lower urinary tracts.

## 4.2 Statement of Hypothesis.

The host's endogenous estrogen levels modulate Dr+ *E. coli* colonization in the urinary tract via ER activation pathway.

## 4.3 Results

### 4.3.1: Estrogen deficiency increases Dr+ *E. coli* colonization in the bladders and kidneys of OVX C3H/HeJ mice

To investigate the effects of estrogen deficiency on Dr+ *E. coli* colonization in the urinary tract, age-matched OVX and Non-OVX mice were subjected to Dr+ *E. coli* induced experimental UTI. Infected mice were euthenized at 2 and 6 days post-UTI induction and colonization in the urinary tracts was evaluated by determining the bacterial load (details in chapter III, sections 3.2.5 and 3.2.6).

Bacterial counts in the bladders of the OVX and the Non-OVX mice at 2 and 6 days post-infection are represented in figures 6A and 6B, respectively. The bacterial colonization in the bladders of OVX mice was significantly higher than the colonization observed in the bladders of Non-OVX mice (Figure 6A and 6B). Notably, the viable bacterial counts from the bladders of OVX mice at 2 and 6 days post-infection remained similar. However, in the Non-OVX mice, bladder colonization at 6 day post-infection was 20-fold lower than the colonization observed at 2 days post-infection, though the difference was not found to be statistically significant (Figure 6C).

Bacterial colonies were recovered from both of the kidneys of all the animals in each of the experimental groups. The bacterial counts obtained from the left and the right kidney of each mouse in the study were found to be comparable for 2, as well as 6 days post-infection, indicating that each kidney was equally infected following UTI induction at both the time points (appendix supplementary Figures 1A-1D). To be consistent, CFU from the left kidneys of mice were used for comparison of bacterial load among the study groups.

Bacterial counts in the kidneys of the infected OVX and Non-OVX mice at 2 and 6 days post-infection are represented in Figures 6D and 6E respectively. Following 2 days of infection, the bacterial colonization in the kidneys of OVX mice was lower compared to that in the kidneys of Non-OVX mice (Figure 6D). In contrast, at 6 days post-infection, colonization in the kidneys of OVX mice was found to be 13-fold higher compared to that in the kidneys of Non-OVX mice (Figure 6E). Although the kidneys of all the mice remain infected at day 6 post-infection, bacterial colonization increased in the OVX mice, but decreased in the Non-OVX mice by 10-fold from 2 to 6 days post-UTI induction (Figure 6F). We have earlier observed that at 21 days post-infection, Dr+ *E. coli* colonization in the kidneys of OVX mice remained significantly higher compared to the Dr+ *E. coli* colonizationt in the kidneys of Non-OVX mice (Figure 6F and preliminary data, Figure 1).

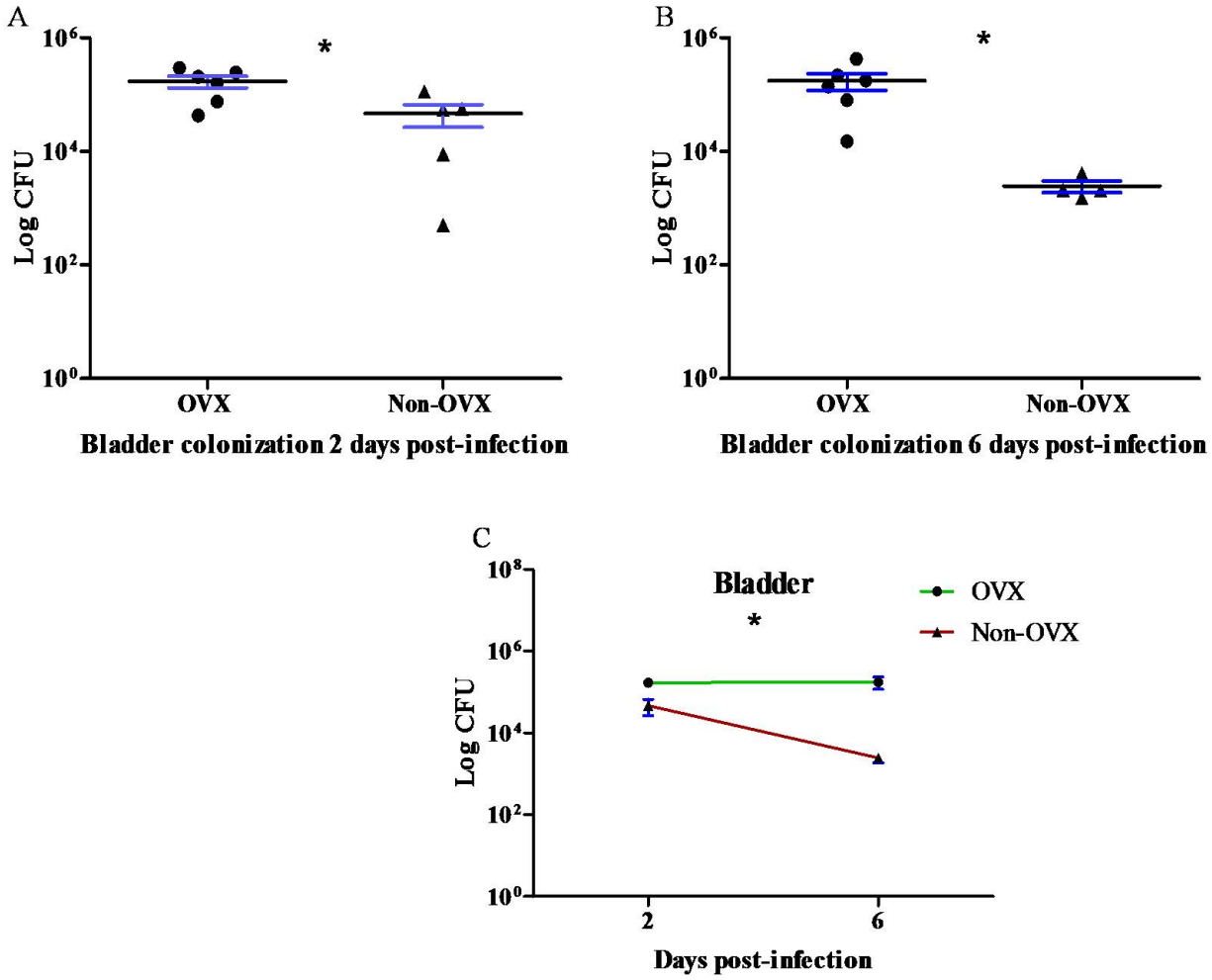
A
B
C
Log CFU
OVX
Non-OVX
Bladder colonization 2 days post-infection
Bladder colonization 6 days post-infection
Bladder
Days post-infection
2
6
*

**Figure 6**

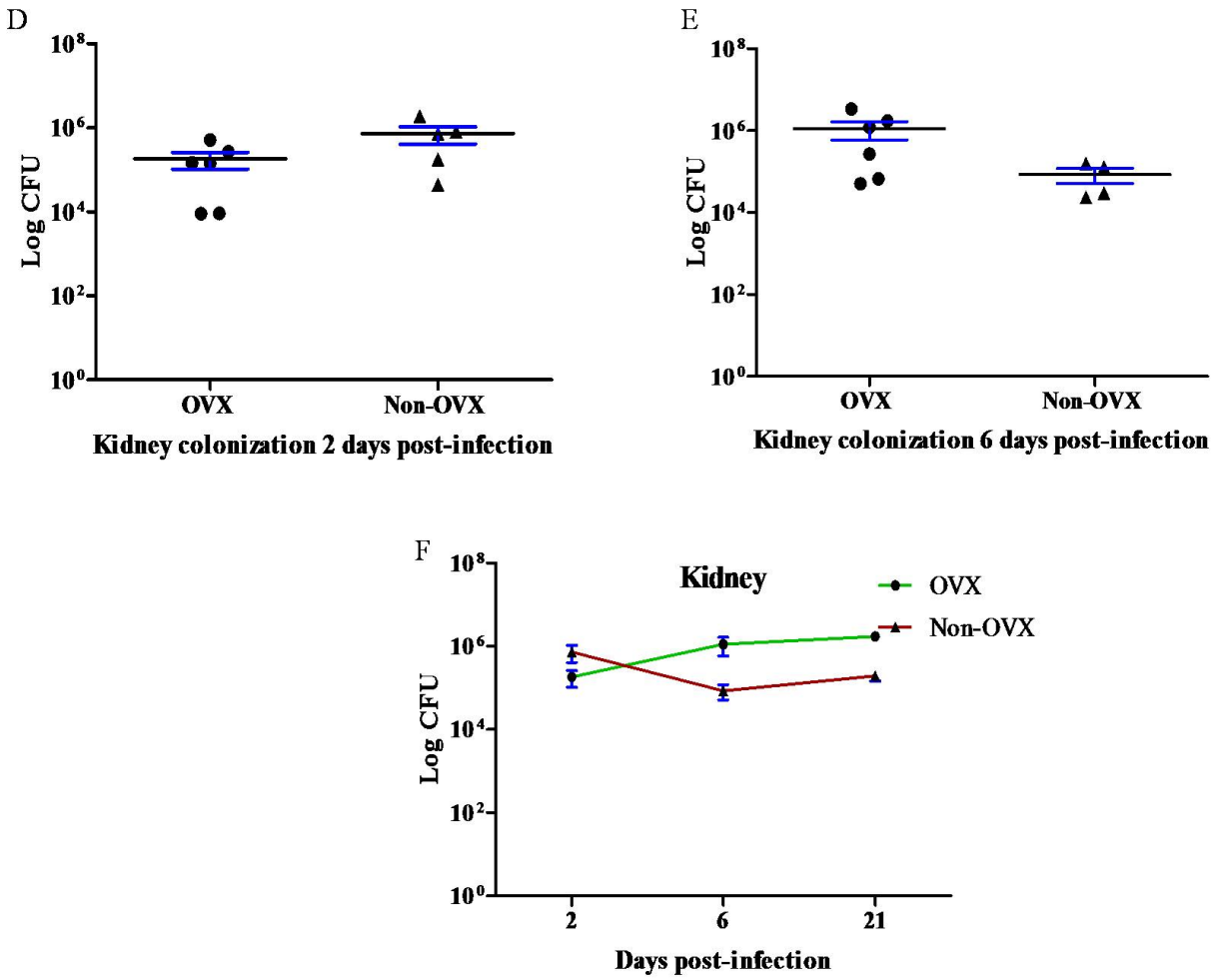


**Figure 6: Bacterial counts in the bladders and kidneys of OVX and the Non-OVX C3H/HeJ mice.** Bacterial counts per gram bladder **(A and B)** and kidney **(D and E)** tissues in OVX and Non-OVX mice following 2 and 6 days post-UTI induction were enumerated and represented as CFU/gm tissue. Bacterial counts from OVX mice are depicted by closed circles and those from Non-OVX mice are indicated by closed triangles. Each symbol in the graphs represents values from a single mouse. The horizontal black bars denote the mean CFU ± SEM from each study group and the error bars represent data from 4-6 mice in each study group. **C)** The bacterial counts in the bladders of OVX mice remained similar at 2 and 6 days post-infection while the bacterial counts in the bladders of Non-OVX mice decreases. **F)** The bacterial counts in the kidneys of OVX mice increased while those in the kidneys of Non-OVX mice decreased during the course of infection. Data were analyzed by Student's t-test where $P<0.05$ was considered significant (*).

### 4.3.2: Estrogen pretreatment decreases Dr+ *E. coli* colonization in the bladders and kidneys of OVX C3H/HeJ mice

To further investigate the effects of a physiological dose of estrogen on colonization of Dr+ *E. coli* in the the mice urinary tracts, we pretreated the OVX mice with a physiological dose of E2 prior to UTI-induction. E2-treated OVX mice attained physiological levels of serum E2 following the treatment, and maintained it at the physiological levels during the experiment (Table 7) (Tai *et al*, 2008). Age matched vehicle- and E2-treated OVX C3H/HeJ mice were induced with Dr+ *E. coli* mediated experimental UTI and the bacterial counts in the bladder and kidney tissues were enumerated at 2 and 6 days post-infection.

**Table 7**: **Serum E2 levels in OVX and E2 treated OVX C3H/HeJ mice**

| **Mice** | **Serum E2 levels (mean ± SEM)** | **Number of mice (n)** |
|---|---|---|
| OVX | 15.40±0.092 pg/ml | n=5 |
| OVX-E2 (2 days post-infection) | 33±1.7 pg/ml ** | n=6 |
| OVX-E2 (6 days post-infection) | 34.17±4.8 pg/ml** | n=6 |
| **E2-treated OVX mice attained physiological levels of E2 which are significantly higher than the E2 levels in untreated OVX mice. | | |

Viable bacterial counts obtained from the bladders of vehicle-and E2-treated OVX mice at 2 and 6 days post-infection are represented in Figures 7A and 7B respectively. Pretreatment with a physiological dose of E2 markedly decreased bacterial colonization in the bladders of OVX mice. The bacterial colonization in the bladders of vehicle-and

E2-treated mice was not significantly different at 2 days post-infection (Figure 7A), but at 6 days post-infection the bladders of vehicle-treated OVX mice were significantly more colonized, showing 47-fold higher bacterial colonization compared to that in the bladders of E2-treated OVX mice (Figure 7B). The bacterial counts in the vehicle-treated mice remained the same for 2, as well as 6 days post-infection, however, the bacterial counts decreased markedly by 30-fold in the E2-treated mice as the infection progressed from 2 to 6 days following UTI induction (Figure 7C), but this difference was not found to be statistically significant.

The incidence of kidney infection among the E2-treated OVX mice was 73% lower compared to the incidence of kidney infection in the vehicle-treated OVX mice at day 6 post-infection, however the reduction was not statistically significant (Figure 7E). At 21 day post- infection, significant reduction (by 60%) in Dr+ *E. coli* colonization was observed in the kidneys of E2-treated OVX mice compared to that in the kidneys of vehicle-treated OVX mice ( Figure 1). Among the OVX mice, colonization in the kidneys at 6, and 21 days post-infection, was higher compared to the colonization at 2 days post-infection, suggesting increase in Dr+ *E. coli* infection with time (Figure 7F). In contrast, the E2-treated OVX mice showed reduced kidney colonization at 6 and 21 days post-infection compared to the colonization at 2 days post-infection, suggesting a gradual clearance of Dr+ *E. coli* infection from the kidneys of E2 treated OVX mice (Figure 7F). Although, the infection in the kidneys of E2-and vehicle-treated OVX mice persisted until 21 days post-infection, the colonization in the kidney of E2-treated mice remained significantly lower compared to that in the kidney of vehicle-treated mice (Figure 7F).

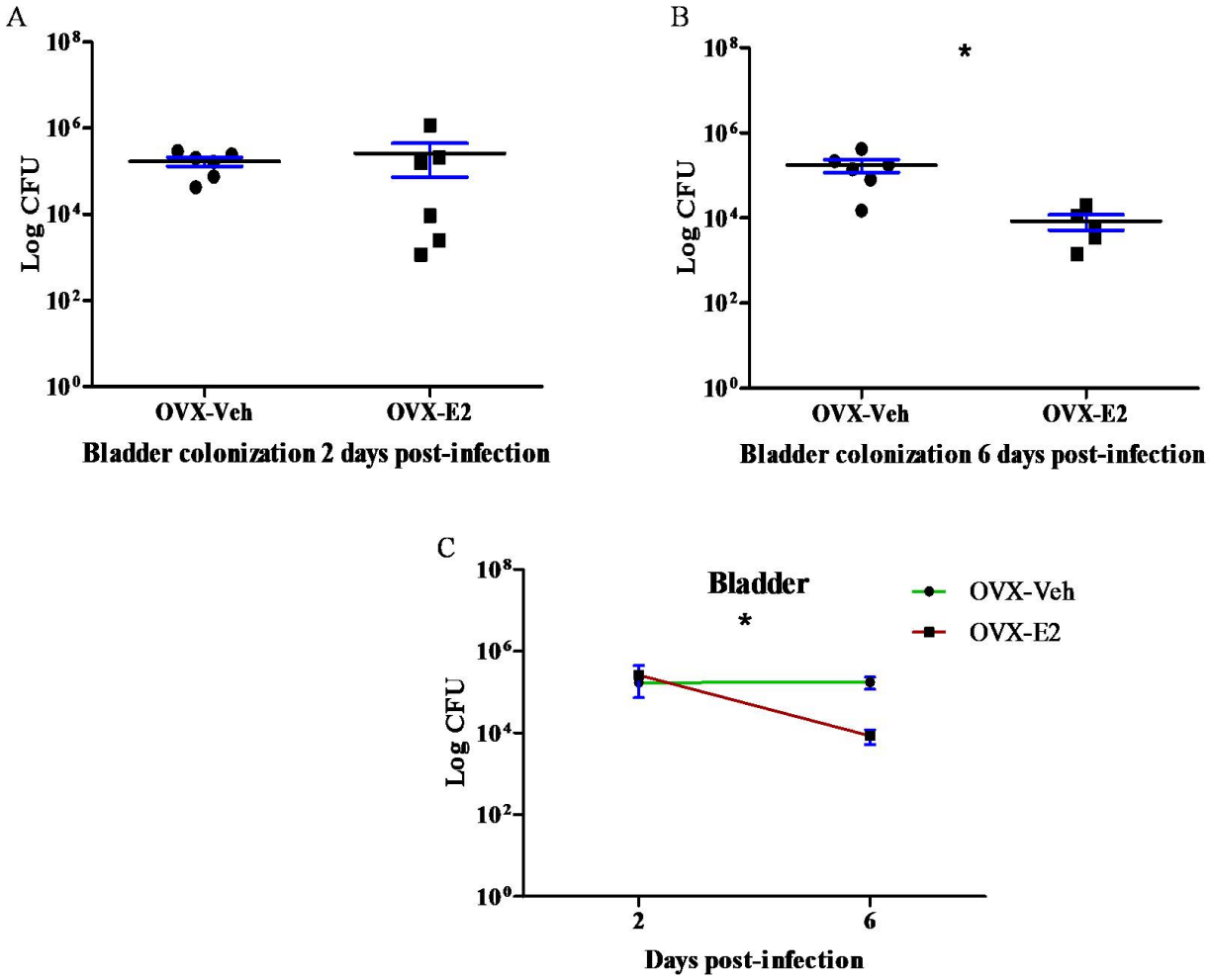
A
$10^8$
$10^6$
$10^4$
$10^2$
$10^0$
Log CFU
OVX-Veh
OVX-E2
Bladder colonization 2 days post-infection
B
*
$10^8$
$10^6$
$10^4$
$10^2$
$10^0$
Log CFU
OVX-Veh
OVX-E2
Bladder colonization 6 days post-infection
C
Bladder
*
OVX-Veh
OVX-E2
$10^8$
$10^6$
$10^4$
$10^2$
$10^0$
Log CFU
2
6
Days post-infection

**Figure 7**

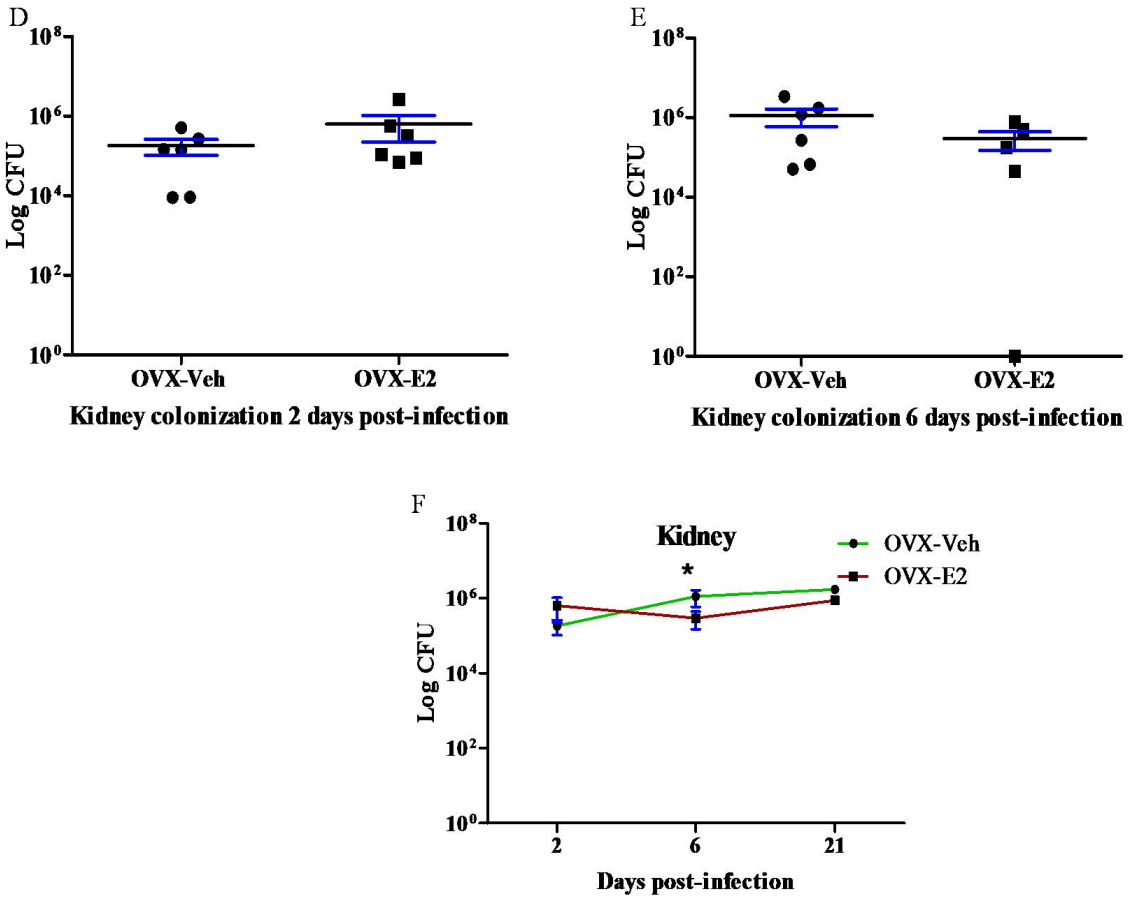


**Figure 7: Bacterial counts in the bladders and kidneys of E2-treated OVX C3H/HeJ mice.** OVX mice were treated with vehicle (Veh) or E2 at a physiological dose prior to experimental UTI induction with Dr+ *E. coli*. Bacterial CFU per gram bladder **(A and B)** and kidney **(D and E)** tissues in mice following 2 and 6 days post-UTI induction are presented in each graph. Bacterial counts from vehicle-treated mice are depicted by closed circles and those from E2-treated-mice are indicated by closed squares. Each symbol in the graph represents values from a single mouse. Tissues with no detectable CFU recovered from the homogenate culture were counted as if 1 CFU was found. The horizontal black bars denote the mean CFU ± SEM from each study group and the error bars represent data from 4-6 mice in each study group. **C)** Bacterial counts in the bladders of vehicle-treated OVX mice remained similar, while those in the bladders of E2-treated OVX mice decreased during the course of infection. **F)** Bacterial counts in the kidneys of vehicle-treated OVX mice increased while those in the kidneys of E2-treated OVX mice decreased during the course of infection. Data were analyzed by Student's t-test where $P<0.05$ was considered significant (*).

### 4.3.3: ER-antagonist pretreatment increases Dr+ *E. coli* colonization in the bladders and kidneys of C3H/HeJ mice

To investigate the role of ERs in estrogen-mediated protective action against Dr+ *E. coli* colonization, age matched ER-antagonist ICI 182, 780- and vehicle-treated Non-OVX C3H/HeJ mice were infected with Dr+ *E. coli* following which bacterial colonization in the bladder and kidney tissues of infected mice was determined at 3 and 7 days post-infection.

At 3 days (Figure 8A), as well as 7days (Figure 8B) post-UTI induction, increased bacterial colonization was found in the bladders of ICI 182,789-treated mice compared to the colonization in the bladders of vehicle-treated mice, however the differences were not statistically significant. The bacterial colonization in the bladders of the vehicle-treated mice decreased markedly at day 7 post-infection suggesting bacterial clearance; however, the bacterial colonization in ICI 182,780-treated mice increased at 7 days post-infection, suggesting bacterial replication and persistence in absence of active ER (Figures 8A and 8B).

At both 3 and 7 days post-infection, bacterial colonization in the kidneys of ICI 182,780-treated mice was comparatively higher than the colonization in the kidneys of vehicle-treated mice (Figure 8C and 8D).

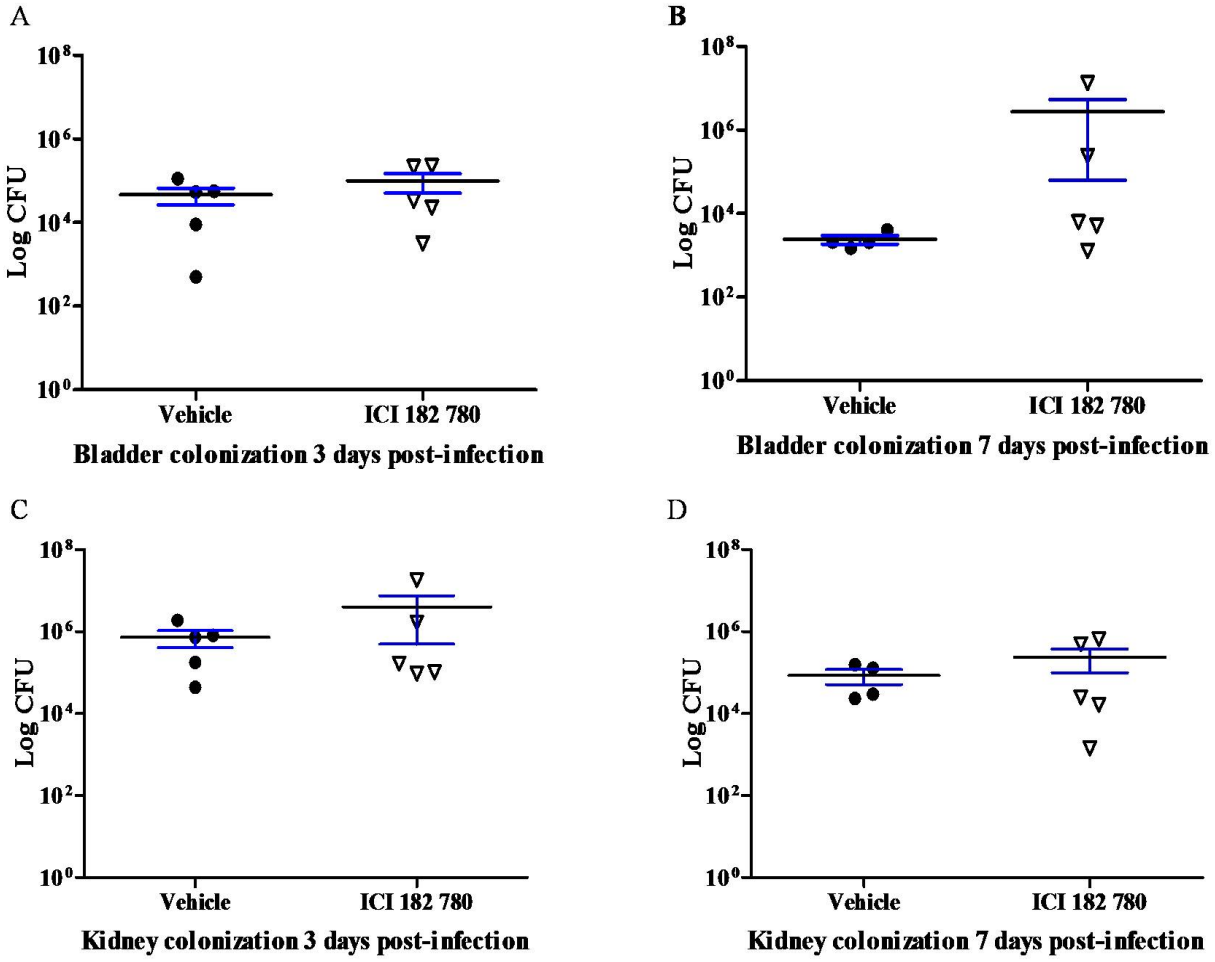
A
B
C
D
Log CFU
$10^8$
$10^6$
$10^4$
$10^2$
$10^0$
Vehicle
ICI 182 780
Bladder colonization 3 days post-infection
Bladder colonization 7 days post-infection
Kidney colonization 3 days post-infection
Kidney colonization 7 days post-infection

**Figure 8**

**Figure 8: Bacterial counts in the bladder and kidneys of ICI 182, 780 treated-Non-OVX C3H/HeJ mice.** Non-OVX mice were treated with ER-antagonist ICI 182, 780 prior to UTI induction. Bacterial CFU per gram bladder **(A-B)** and kidney **(C-D)** tissue from vehicle-and ICI 182, 780-treated Non-OVX mice, at 3 and 7 days post-UTI induction are represented in each graph. Bacterial CFU counts from vehicle-treated mice are depicted by closed circles and those from ICI 182 780 treated mice are indicated by open triangles. Each symbol in the graphs represents a value from a single mouse. The horizontal black bars denote the mean CFU ± SEM from each study group and the error bars represent data from at least 5 mice. Data were analyzed by Student's t-test for Figure 8A- 8D where $P<0.05$ was considered significant.

### 4.3.4: Dr+ *E. coli* colonization in the bladders and kidneys of ERα-/- mice is higher compared to the colonization in the ERα+/+ mice

The mRNA levels of ERα were found to be significantly higher than those of ERβ in both the bladders and the kidneys of ERα+/+ B6.129 female mice (Figure 9A). We investigated the specific role of ERα in determining the protective effects of estrogen against Dr+ *E. coli* colonization using ERα-/- and ERα+/+ female B6.129 mice.

Bacterial colonization were determined in the bladders and kidneys of ERα-/- and ERα+/+ mice following 3 and 7 days post-UTI induction. We could not culture any bacteria from the bladder tissue homogenates of 3 day infected mice. The bladder tissue sections that were used for culture were very small ($1/3^{rd}$ of the bladder). It is thus possible that we were not able to detect any infection due to the small size of the tissue section. Assuming that $1/3^{rd}$ section of bladder is too small for bacterial culture; we used one half of bladder for determining the bacterial counts in the bladder tissues from the mice infected with Dr+ *E. coli* for 7 days.

The bacterial colonization in the bladders and kidneys of ERα-/- and ERα+/+ mice are represented in the Figures 9B-9D. At 7 days post-infection, the bladders of ERα-/- mice had significantly increased bacterial colonization, showing 28-fold higher colonization compared to that in the bladders of ERα+/+ mice (Figure 9B). At both 3 (Figure 9C), and 7 days (Figure 9D) post-infection the bacterial colonization in the kidneys of ERα-/- mice were also found to be higher compared to that in the kidneys of ERα+/+ mice

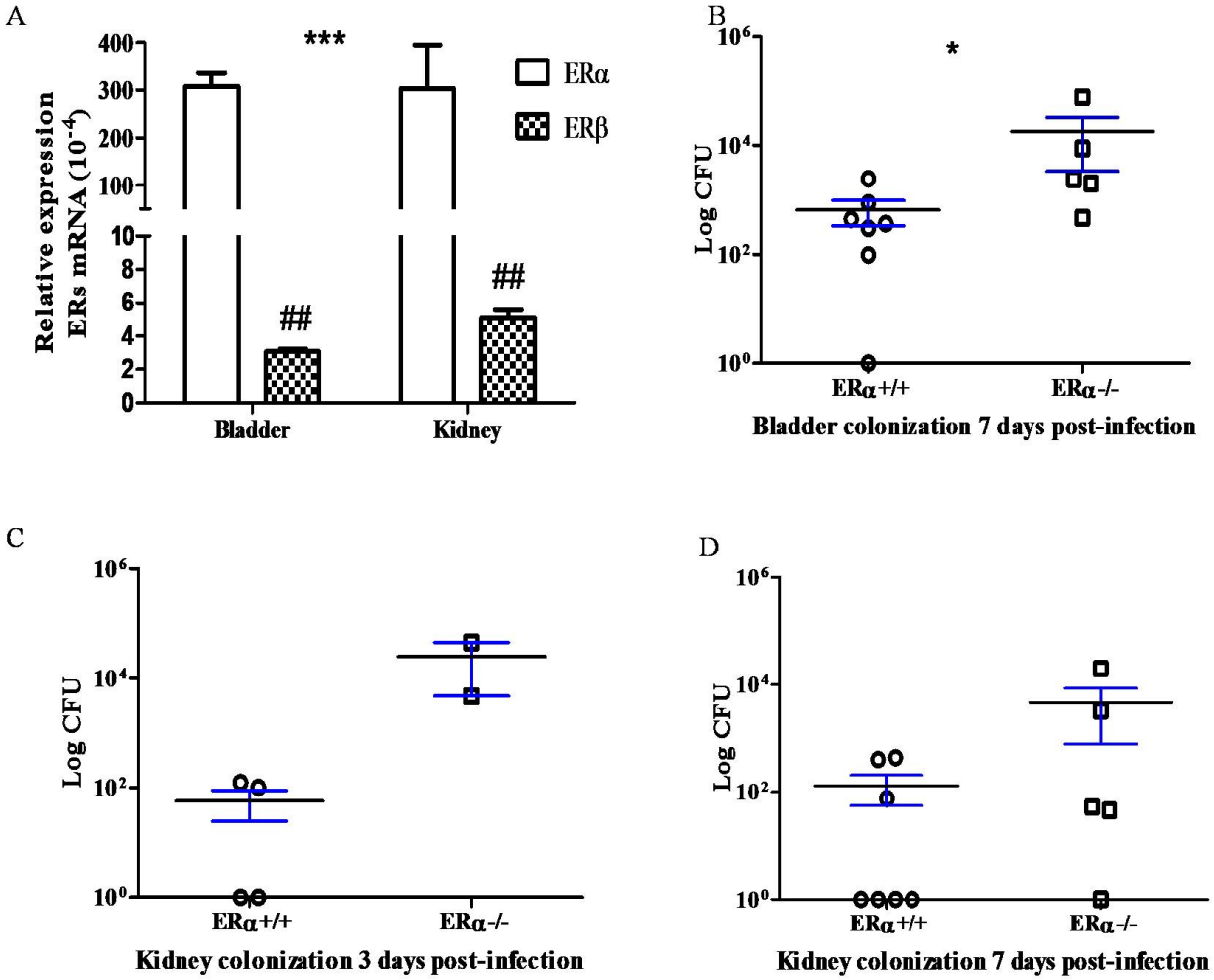
A
Relative expression ERs mRNA ($10^{-4}$)
400
300
200
100
10
8
6
4
2
0
***
##
##
ERα
ERβ
Bladder
Kidney
B
Log CFU
$10^6$
$10^4$
$10^2$
$10^0$
*
ERα+/+
ERα-/-
Bladder colonization 7 days post-infection
C
Log CFU
$10^6$
$10^4$
$10^2$
$10^0$
ERα+/+
ERα-/-
Kidney colonization 3 days post-infection
D
Log CFU
$10^6$
$10^4$
$10^2$
$10^0$
ERα+/+
ERα-/-
Kidney colonization 7 days post-infection

**Figure 9**

**Figure 9: A) ERα and ERβ mRNA levels in the bladders and kidneys of ERα+/+ mice**. ER subtype mRNA levels were analyzed by SYBR green quantitative real-time RT-PCR using ΔCT method with gene specific primers. Target gene expression was normalized relative to PPIA exprssion in each animal. Expression values are presented as mean relative expression ± SEM from 2 mice. ERα mRNA were found to be predominant in bladders, as well as kidneys (##, $P<0.005$). No significant difference was observed in the ERα and ERβ mRNA levels between bladders and kidneys. Data were analyzed by Two-way ANOVA, followed by Bonferroni post hoc test for multiple comparisons where $P <0.05$ was considered significant.

**B-D) Bacterial counts in the bladders and kidneys of ERα-/- and ERα+/+ mice.** Bacterial CFU per gram bladder (B) and kidney (C and D) tissues in ERα-/- and ERα+/+ mice following 3 and 7 days post-UTI induction are represented. The Bacterial counts from ERα+/+ mice are depicted by open circles and those from E2-treated mice are indicated by open squares. Tissues with no detectable CFU recovered were counted as if 1 CFU was obtained. Each symbol in the graphs represents a value from a single mouse. The horizontal black bars denote the mean CFU ± SEM from each study group and the error bars represent data from 2-7 mice in each study group. Data were analyzed by Student's t-test where $P<0.05$ was considered significant (*).

## 4.4 Discussion

Estrogen exerts multiple actions on the female urinary tract; however, there is limited information regarding the effects that estrogen has on bacterial colonization in the host urinary tract. In the present study, we demonstrate that estrogen deficiency induced by ovariectomy resulted in marked increase in the colonization of Dr+ *E. coli* in the bladders, as well as kidneys of OVX mice, compared to that in the estrogen-sufficient Non-OVX mice. Subcutaneous administration of E2 at a physiological dose in the OVX mice induced a definite reduction in the levels of bacterial colonization in the bladders and kidneys, suggesting a protective action of the physiological dose of estrogen against Dr+ *E. coli* colonization. From these results it is evident that following UTI induction, the mice with circulating physiological estrogen levels start clearing the Dr+ *E. coli* infection from bladders as well as kidneys. In contrast, mice with estrogen deficiency cannot clear the infection during initial stages following UTI induction, and so are unable to control bacterial replication, therefore infection increases and persist in these mice. Thus, it appears that under the conditions in which these experiments were performed, estrogen deficiency increases, while physiological estrogen levels protect against Dr+ *E. coli* colonization in the the mice urinary tractss. Our result is consistent with the study by Ruiz *et al* that also reported decrease in levels of UPEC colonization in the urinary tracts of mice treated with E2 (de Ruiz *et al*, 2001). Furthermore, using a rat uterus model, it has been previously shown that *E. coli* adhered to the epithelium of OVX rats, causing purulent endometritis, but the OVX mice treated with physiological E2 levels *did not* show *E. coli* adherence to the epithelium (Nishikawa, 1985). Additionally, several clinical studies have suggested that decreased estrogen levels in postmenopausal females

may be an important contributing factor leading to increased UTI susceptibility, and that estrogen replacement ( local and /or systemic) therapy provides protective effects against UTIs in these women (Hextall, 2000, Oliveria *et al*, 1998, Perrotta *et al*, 2008).

Interestingly, a similar study reported an increase in the rate of Dr+ *E. coli* and other UPEC infections, in the kidneys of OVX C3H/HeJ mice following treatment with a pharmacological dose of E2 (Curran *et al*, 2007). The contrasting results from Curran *et al* and our study may be due to differences in the doses of E2 administered to the experimental animals. The dose of E2 used in our study is comparable to the physiological estrogen range found in the host (6μg/kg/day administered for 5 days results in serum estrogen levels of 35 pg/ml), whereas, the total dose of E2 used in the study by Curran *et al* was very high (21 day release 0.25mg E2 pellets were implanted that resulted in serum estrogen levels of 800 pg/ml). Pregnancy, during which estrogen levels are elevated, is also associated with higher incidence of UTIs. Moreover, epidemiological studies have indicated that over 30% of pregnant women with pyelonephritis are colonized by UPEC expressing the Dr adhesins (Servin, 2005). Notably, a group studying the effects of estrogen therapy in older females reported that the postmenopausal females receiving long term estrogen therapy have 2-fold higher risk for acquiring UTIs than do the control patients (Orlander *et al*, 1992). The effects of E2 on bacterial colonization in the urinary tract appear to be complex and may be dose-dependent. Based on our results and the information available in the literature, it appears that estrogen at physiological doses have beneficial effects in the hosts against UTIs;

while, pharmacological E2 doses and long-term estrogen therapy may contribute to increased susceptibility to UTIs.

Estrogen acts through ERs that are expressed throughout the urinary tract. In this study, we have demonstrated that antagonizing ER activation with anti-estrogen ICI 182,780 in Non-OVX C3H/HeJ mice leads to abolition of the estrogen-protective effects resulting in increased Dr+ *E. coli* colonization in the bladders and kidneys of the infected mice. Thus, it is evident that the estrogenic protection in the the mouse urinary tract against Dr+ *E. coli* colonization is mediated through ER-activation. Furthermore, we found increased susceptibility of ERα-/- mice to Dr+ *E. coli* colonization compared to that in the ERα+/+ mice, indicating a functional role of ERα in providing estrogenic protection against Dr+ *E. coli* colonization in the urinary tracts of the female mice. Protective effects of estrogen via ERα activation against infection have also been reported by other studies. For example, estrogen via ERα activation is found to be protective against *Listeria monocytogen* infection in female mice (Yeretssian *et al*, 2009). Interestingly, treatment of female mice with higher doses of E2 was found to increase *Listeria monocytogen* infection (Pung *et al*, 1984). In addition, ERα gene polymorphisms have been found to be associated with HBV infection in the Chinese population (Deng *et al*, 2004,). To date, there are no direct studies reporting the specific role of ERα or ERβ in estrogen action on the susceptibility to UTIs. Our study provides the first *in vivo* evidence for ERα mediated estrogenic protection against Dr+ *E. coli* infection in the mouse kidney. Furthermore, our results indicate that female populations carrying mutations or polymorphisms in ERα gene may be more prone to UTIs.

The female genitourinary tract is known to be sensitive to the effects of estrogen (Robinson & Cardozo, 2004), however the precise mechanisms by which estrogen affects susceptibility to UTIs remain to be fully characterized. Moreover, from the view of numerous reports that have studied the effects of estrogen on UTI susceptibility in females, it appears that these effects may be multi factorial (Ahmed *et al*, 2008, Hextall, 2000, Hooton, 2000, Jelinsky *et al*, 2008). While several clinical studies have documented that estrogen replacement therapy (local topical application/ oral administration) lowers the risk of UTIs in the postmenopausal women, some studies either did not find any therapeutic benefits of estrogen replacement, or reported it to increase the rate of UTIs in females receiving the therapy (Hextall, 2000, Molander, 1993, Oliveria *et al*, 1998, Orlander *et al*, 1992, Perrotta *et al*, 2008, Stern *et al*, 2004, Xu *et al*, 2001). The conflicting evidences regarding the effectiveness of estrogen for prophylaxis against recurrent UTIs in the postmenopausal women exist because the optimum doses of therapy, route of delivery, and duration of therapy are yet to be determined. Moreover, precise molecular mechanisms associated with estrogenic effects on female genitourinary tract are not completely understood.

In premenopausal women, the circulating estrogens promote lactobacilli colonization in the vagina by regulating glycogen metabolism, resulting in low vaginal pH that inhibits the growth of potential uropathogens. In the postmenopausal females, estrogen deficiency causes reduced glycogen and disappearance of lactobacilli from the vaginal flora, resulting in increased vaginal pH. This promotes vaginal colonization by members

of Enterobacteriaceae, predominantly *E. coli* and increases the risk of UTIs in the postmenopausal women. Furthermore, low estrogen levels in the postmenopausal women also contribute to local mucosal atrophy and reduction in mucin secretion resulting in increased risk for UTIs. Estrogen replacement therapy in postmenopausal women at low doses have been shown to restore the vaginal flora and pH and greatly reduce the risk of vaginal atrophy, thus providing protection against recurrent UTI (Cotreau *et al*, 2007, Hextall, 2000, Raz, 2001b, Rozenberg *et al*, 2004).

Our results show that estrogen and ERα activity play a definite role in Dr+ *E. coli* colonization in the bladder and kidney of the female mouse. Estrogen may induce multiple effects on reducing the risk of UTIs, and the possible mechanisms may include modulation of colonization receptor expression, regulation of bacterial internalization and induction of inflammatory responses during infection. Elucidating the cellular and molecular mechanisms behind ERα-mediated enhanced estrogenic protection against Dr+ *E. coli* induced UTI will have broad clinical and experimental implications. Results of the present study illustrate the potential effects of estrogen deficiency and physiological E2 levels as well as role of ERα in modulation of Dr+ *E. coli* colonization in the urinary tract during the course of experimentally induced UTI. We next investigated the effects of estrogen on bacterial internalization *in vitro* in mIMCD3 cells.

# CHAPTER V

# ESTROGEN MODULATES Dr+ *ESCHERICHIA COLI* INVASION IN THE MOUSE INNER MEDULLARY COLLECTING DUCT (mIMCD3) CELLS VIA ESTROGEN RECEPTOR ACTIVATION

## 5.1 Introduction

UTIs have typically been considered extracellular infections of the urinary tract. However, recent reports from animal as well as clinical studies, have demonstrated invasion of nonphagocytic uroepithelial cells by UPEC (Bower *et al*, 2005, Mulvey, 2002, Rosen *et al*, 2007). Following attachment, invasion of UPEC into host cells is recognized as a crucial event during UTI pathogenesis. Invasion allows the UPEC to colonize, multiply, disseminate, and persist within the host for a long period of time. In addition, invasion into the host epithelium facilitates UPEC evasion from the innate immune defense mechanisms, neutrophil clearance, and microbicidal effects of antimicrobial peptides and antibiotics in the host's urinary tract (Blango & Mulvey, 2010, Dhakal *et al*, 2008, Kerrn *et al*, 2005, Mulvey *et al*, 2000, Mulvey *et al*, 2001). Scanning and electron micrographs of infected tissues have shown the presence of internalized UPEC either in the endocytic vesicle or free within the cytoplasm of infected cells (Goluszko *et al*, 1997, Kerrn *et al*, 2005, Mulvey *et al*, 2000).

Following invasion, the bacteria are able to reside and multiply in an endocytic vesicle within the cells forming the quiescent reservoirs of UPEC called intracellular bacterial communities. They are then effluxed, only to adhere and invade the adjacent host's cells, thus maintaining the infectious cycle within the host urinary tract (Mulvey *et al*, 2000, Mulvey, 2002, Rosen *et al*, 2007).

Dr+ *E. coli*, a pyelonephritic UPEC and has been shown to be internalized by several epithelial cell lines following its attachment to its receptor DAF which is expressed on the apical surface of the cells (Das *et al*, 2005, Fang *et al*, 2004, Goluszko *et al*, 1997, Goluszko *et al*, 2001, Guignot *et al*, 2000, Guignot *et al*, 2001, Kerneis *et al*, 1994, Zalewska-Piatek *et al*, 2009). Invasion of Dr+ *E. coli* in mIMCD3 cells has not been previously reported. Although some studies have been conducted to elucidate the role of estrogen on bacterial adhesion onto the uroepithelium, no direct or indirect role of estrogen and ERs on regulating bacterial invasion in urpoepithelial cells has been previously described.

Results from our *in vivo* experimental UTI mouse model indicate that E2 treatment at physiological levels decreases Dr+ *E. coli* colonization in the mouse kidney and this estrogen-mediated protection is ERα-dependent. Dr+ *E. coli* has been shown to adhere to human kidney collecting duct cells (Virkola *et al*, 1988). Our preliminary *in vitro* experiments have demonstrated that E2 treatment at physiological levels decreased Dr+ *E. coli* invasion in human hepatocytes and Huh7 cells a liver cell line. The present study was conducted to elucidate the Dr+ *E. coli* invasion in the mouse kidney collecting duct

mIMCD3 cells to determine and verify our previous observations on protective effects of E2 treatment against Dr+ *E. coli* invasion *in vitro*. In this study, we have also characterized the functional roles of ER subtypes, ERα and ERβ, in mediating E2 effects during Dr+ *E. coli* invasion in mIMCD3 cells. This was achieved by determining Dr+ *E. coli* internalization in mIMCD3 cells treated with ERα and ERβ selective ligands. The compound PPT displays 410-fold more selectivity for ERα over ERβ and is used as ERα selective agonist, and DPN exhibits a 70-fold higher binding affinity for ERβ than ERα; therefore, it is used as ERβ selective agonist (Meyers *et al*, 2001, Stauffer *et al*, 2000). We antagonized the ERs with ICI 182, 780 and ER subtypes with specific antagonists MPP and R, R-THC to further confirm the role of ERα and ERβ respectively. We report here that E2 at physiological doses reduces Dr+ *E. coli* invasion in mIMCD3 cells while E2 deficiency and a pharmacological E2 dose increases bacterial invasion. Our study indicates that ERα plays a predominant role in estrogen-mediated protection against Dr+ *E. coli* invasion.

## 5.2 Statement of Hypothesis

Estrogen via ERα activation regulates invasion of Dr+ *E. coli* in the kidney tubular cells and thus determine the host susceptibility to Dr+ *E. coli* induced ascending UTI.

## 5.3 Results

### 5.3.1: E2 treatment differentially modulates Dr+ *E. coli* invasion in mIMCD3 cells in a dose-dependent manner

We analyzed the expression of DAF in the mIMCD3 cells at the mRNA, and protein levels, to determine the susceptibility of mIMCD3 cells for Dr+ *E. coli* colonization and internalization (Figure 10 A and 10B). We also examined the cells for ER subtype expression at the mRNA and protein levels (Figure 11A-C). DAF, ERα, and as ERβ were found to be expressed in mIMCD3 cells. Similar to the ER subtype expression pattern in the mouse kidney, we found more ERα mRNA than ERβ in mIMCD3 cells.

To elucidate the effect of E2 on Dr+ *E. coli* infection, mIMCD3 cells were either left untreated or were pretreated with E2 at different non-toxic doses (1, 10, 50 and 100 nM) for 24hrs prior to infection. Drug toxicity for E2 treatment in the cells was determined by MTT assay (appendix supplementary Figure 2A). E2-treated, as well as untreated control cells were incubated with Dr+ *E. coli* for 2 hrs, following which the amount of bacterial infection (determined as bacteria adhered and bacteria invaded) in the cells was enumerated (details in chapter 3 sections 3.3.2 and 3.3.3). Amount of Dr+ *E. coli* invasion in the cells was determined by gentamicin protection assay following 2 hrs of infection. Percentage CFU infection and invasion in the E2-treated cells relative to untreated control cells were calculated.

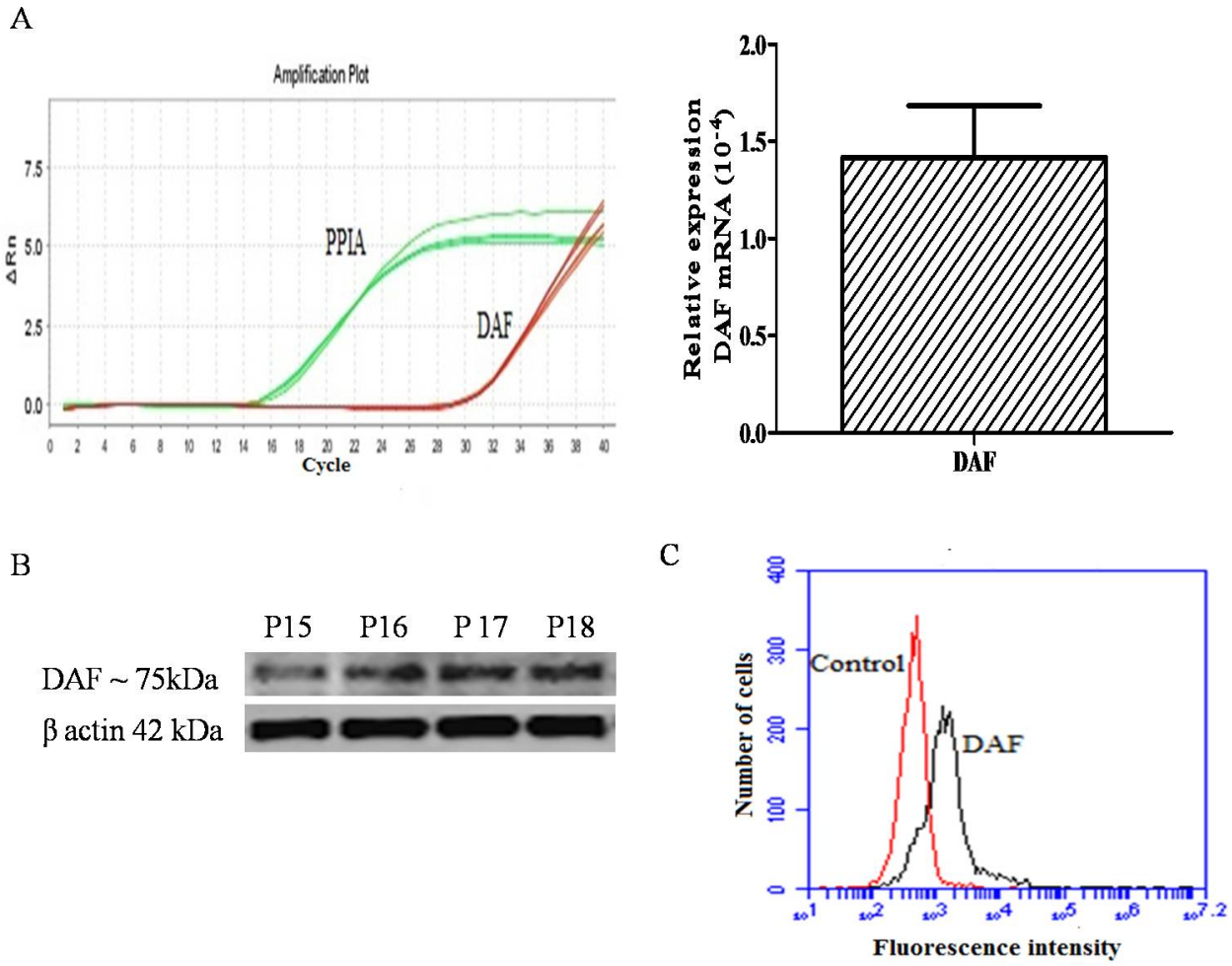


**Figure 10**: **DAF expression in the mIMCD3 cells**. **A)** The DAF mRNA levels were determined by quantitative SYBR green real-time RT-PCR. Amplification plot shows DAF and PPIA expression in mIMCD3 cells. DAF mRNA levels were normalized relative to PPIA mRNA levels in each sample. Expression values are mean relative expression ± SEM from 4 different passages (p 15-p18) of mIMCD3 cells. **B)** Protein expression of DAF in mIMCD3 cells detected using western blot. Total mIMCD3 cell lysate from four different passages were blotted using rat anti-mouse DAF mAb (2C6) and β-actin was blotted as a loading control. **C)** Surface expression of DAF protein on mIMCD3 cells determined by flow cytometry. The area marked by the red boundary represents unstained control cells and the area marked by the black boundary represents cell population stained with PE labeled, hamster anti-mouse DAF mAb (RIKO 5); the same gate was applied for analyzing control and the RIKO 5 stained cell population.

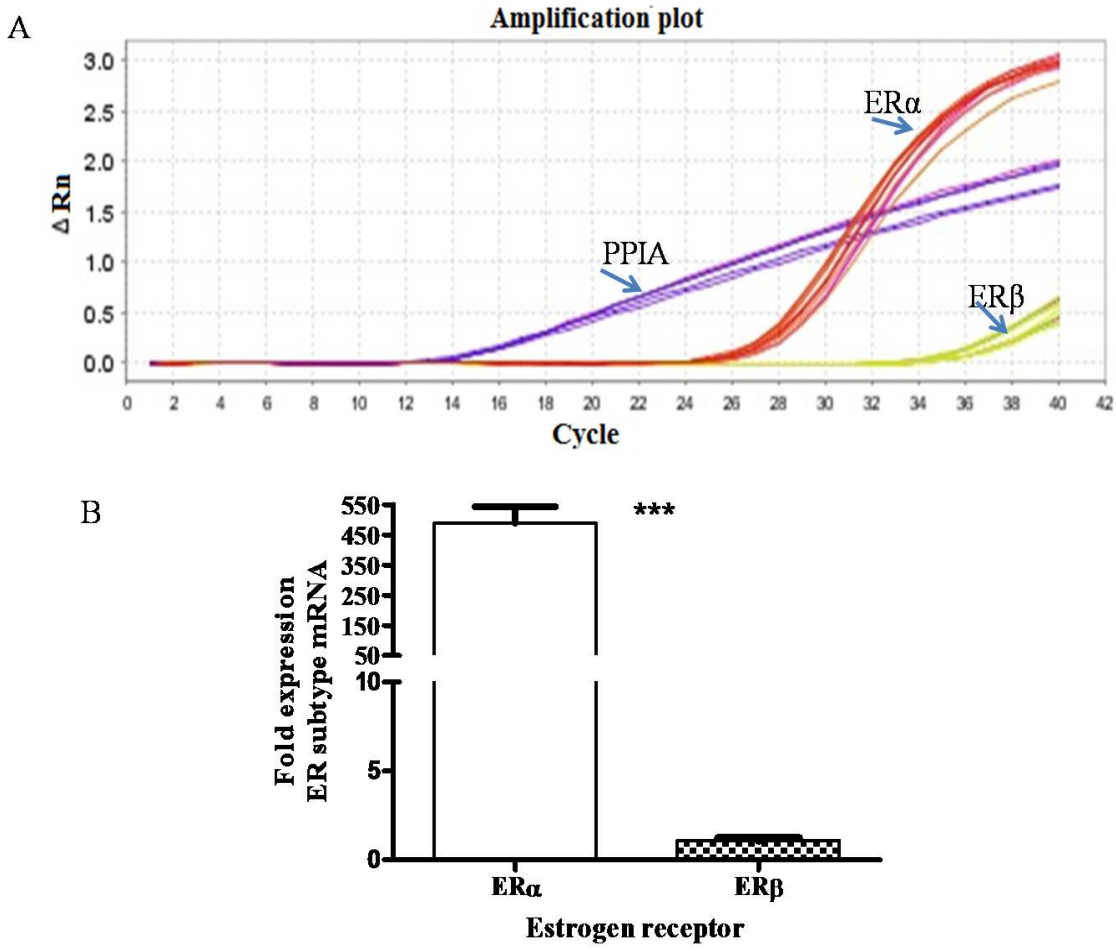


**Figure 11**: **ERα and ERβ expression in the mIMCD3 cells**.

**A) ERα and ERβ mRNA levels in the mIMCD3 cells.** ERα and ERβ mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Amplification plot shows the expression of ER subtypes and PPIA in mIMCD3 cells. ERα and ERβ mRNA levels were normalized relative to PPIA mRNA levels in each sample. Expression values are mean relative expression ± SEM and error bars represents data from 4 different passages (p 15-p18) of mIMCD3 cells. Data were analyzed by Student's t test where $P < 0.05$ was considered significant (*).

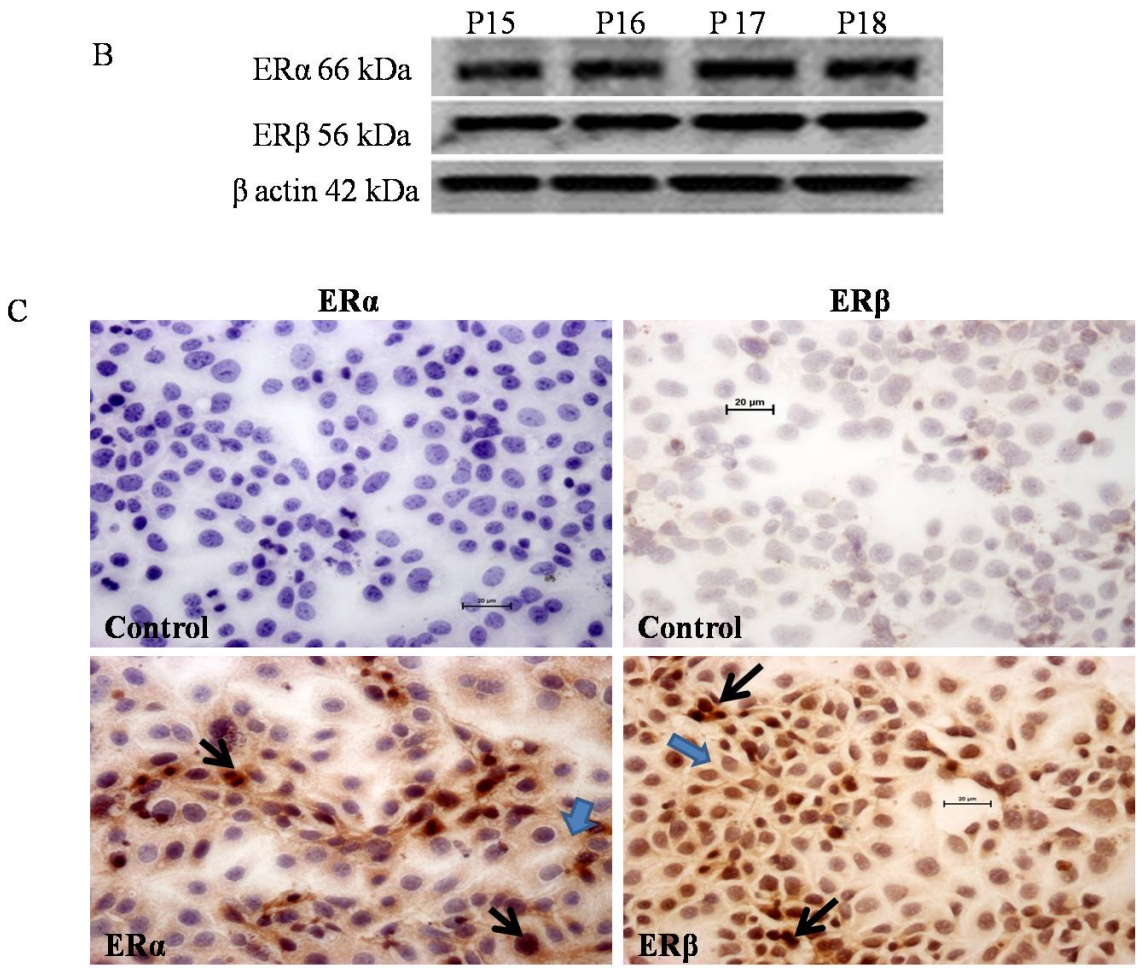


**Figure 11**: **ERα and ERβ expression in the mIMCD3 cells**.

**B) and C) Protein expression of ERα and ERβ in the mIMCD3 cells**. The ERα and ERβ proteins were detected using western blot and immunocytochemistry with ERα and ERβ specific antibodies. **B)** Total mIMCD3 cell lysate from four different passages were blotted using ERα (MC-20) or ERβ (H-150) specific antibodies, β-actin was blotted as a loading control. **C)** Cells grown in chamber slides were stained with ERα (MC-20) or ERβ (PPG5/10) specific antibodies. Negative control images represent hematoxyline-eosine staining of the cells done in absence of the target specific antibodies. In the Figure C black arrows are pointing to cells showing ER staining in cytoplasm, as well as in nucleus and blue arrows are pointing to the cells with only cytoplasmic staining. Scale bar of 20μm applies to all the images in Figure C.

Pretreatment of mIMCD3 cells with physiological doses of E2 (1-10 nM) significantly reduced Dr+ *E. coli* infection (Figure 12A), as well as cellular invasion (Figure 12B). Maximum reduction in bacterial infection, as well as invasion, was observed at a 10 nM E2 treatment. Overall, 40-60 % reduction in Dr+ *E. coli* infection as well as invasion was observed in mIMCD3 cells treated with 10 nM E2 in different experiments. The similarity observed in the amount of bacterial infection and bacterial invasion in mIMCD3 cells implies that bacterial invasion is proportional to bacterial adherence. A significant increase in bacterial invasion was observed in cells treated with pharmacological E2 dose (100 nM), compared to the cells treated with E2 at physiological (1-10 nM E2) doses (Figure 12A and 12B).

We also determined the effect of E2 on Dr+ *E. coli* internalization in hIMCD cells. We confirmed the mRNA expression of DAF by SYBR green real-time RT PCR and ER subtypes by TaqMan real-time RT-PCR; the protein levels of DAF were detected using western blot (data not shown). Dr+ *E. coli* invasion in hIMCD cells treated with different doses of E2 (0, 1, 10, and 50 nM) was determined post 2 hrs of infection. MTT test was performed to determine the E2 toxicity in hIMCD cells (appendix supplementary Figure 2B). Similar to the observations in mIMCD3 cells, Dr+ *E. coli* internalization in hIMCD cells was found to be significantly reduced in cells treated with E2 at physiological doses (Figure 12C).

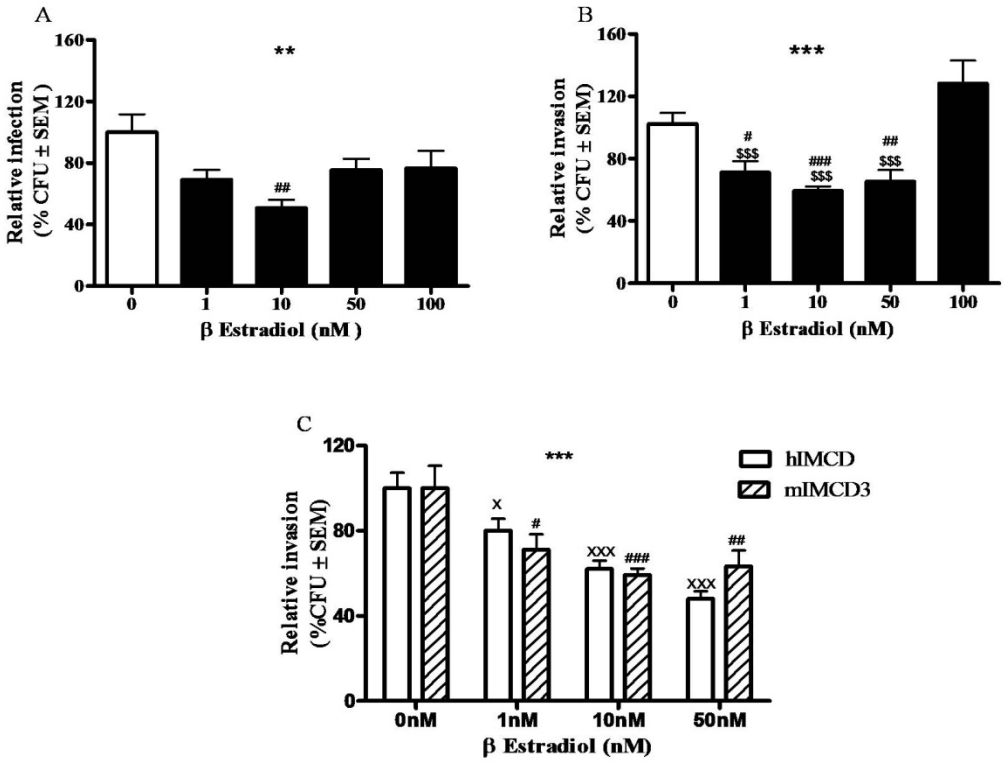


**Figure 12**: **Relative infection and invasion of Dr+ *E. coli* in E2-treated cells. A**) Relative bacterial infection in E2-treated mIMCD3 cells following 2 hrs of incubation with Dr+ *E. coli*. Total infection represents bacteria adhered and internalized into the cells. Infection values are mean bacterial CFU ± SEM expressed as % infection in E2-treated cells relative to untreated cells (considered as 100%). The error bars represent data from at least 2 experiments performed in triplicate. **B**) Relative bacterial invasion in E2-treated mIMCD3 cells following 2 hrs of Dr+ *E. coli* infection determined by gentamicin protection assay in mIMCD3 cells. **C)** Relative bacterial invasion in E2-treated mIMCD3 and hIMCD cells following 2 hrs of Dr+ *E. coli* infection. Invasion values are mean bacterial CFU ± SEM expressed as % invasion in treated cells relative to untreated cells (considered as 100%). The error bars represents data from 6 experiments for Figure B and at least 3 experiments for Figure C, each performed in triplicate. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc test for multiple comparison where $P<0.05$ was considered significant. The character, * indicates significant differences among the treatment groups, # indicates significant difference relative to control treatment in mIMCD3 cells, x indicates significant difference relative to control treatment in hIMCD cells, $ indicates significant difference relative to 100 nM E2 treatment in Figure B. (Physiological E2 dose:1-10 nM).

### 5.3.2: ER-antagonist treatment reverses E2-mediated protection against Dr+ *E. coli* invasion in mIMCD3 cells

The role of ERs in E2 action against bacterial invasion was tested by blocking ER activation with ICI 182, 780. We co-treated mIMCD3 cells with 10 nM E2 (protective physiological dose) and various doses of ICI 182, 780 (0, 0.1, 1 and 10 μM) for 24 hrs prior to infection with Dr+ *E. coli*. The cells were also treated with ICI 182, 780 alone at the above defined doses. The MTT assays were performed to determine the toxicity of ICI 182, 780 at these doses in mIMCD3 cells (appendix supplementary Figure 2C). Cells were infected and bacterial invasion was determined.

A significant increase in bacterial invasion in a dose dependent manner was observed in ICI 182, 780 and E2 co-treated cells compared to cells treated with E2 alone. Treatment with ICI 182, 780 significantly reversed the protective effect of E2 against Dr+ *E. coli* invasion in the cells. Maximal reversal of E2 meditated protection by ICI 182,780 was observed at a dose of 10 μM (Figure 13 A). Bacterial invasion in cells treated with only ICI 182, 780 was comparable to that in control untreated cells and was significantly higher than in cells treated with 10 nM E2 (Figure 13B).

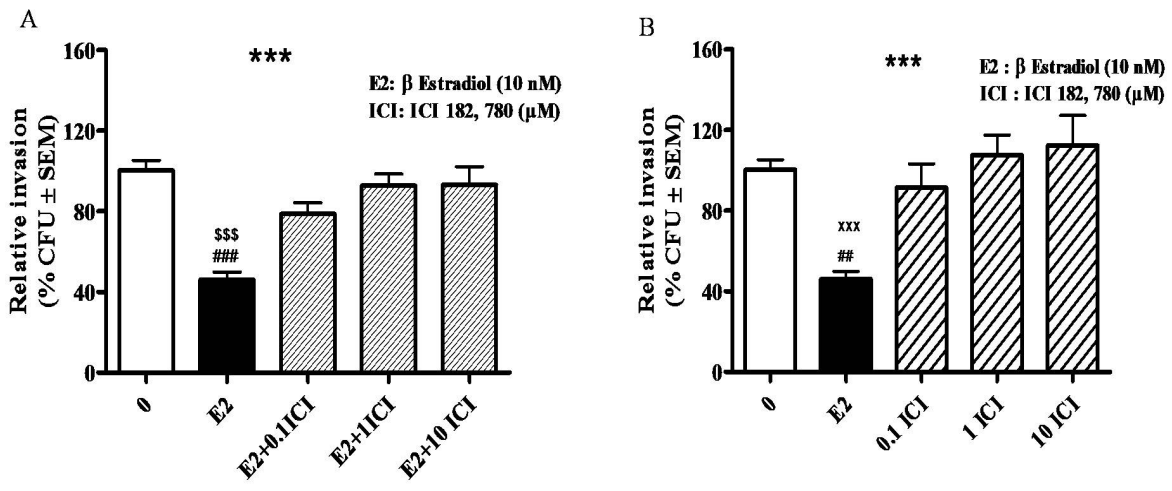


**Figure 13: Relative invasion of Dr+ *E. coli* in ICI 182, 780-treated cells.** Relative bacterial invasion following 2 hrs of Dr+ *E. coli* infection in **A)** ICI+E2 co-treated and **B)** ICI alone treated mIMCD3 cells compared to control untreated cells, as determined by gentamicin protection assay. Invasion values are mean bacterial CFU ± SEM expressed as % invasion in treated cells relative to untreated cells (considered as 100%). The error bars represent data from 6 independent experiments performed in triplicate. Data were analyzed by One-Way ANOVA followed by Tukey's test for multiple comparisons where $P<0.05$ was considered significant. The character, * indicates significant differences among the treatment groups, # indicates significant difference relative to control treatment, $ indicates significant difference relative to E2+ICI co-treatment and x indicates significant difference relative to ICI alone treatment. (Physiological E2 dose1-10 nM)

### 5.3.3: E2 mediates protection against Dr+ *E. coli* invasion in mIMCD3 cells primarily via ERα activation pathway

To investigate the specific role of each ER subtype in estrogen-mediated protection against during Dr+ *E. coli* invasion, we determined the effect of ERα- and ERβ-specific agonists and antagonists treatment on bacterial internalization in mIMCD3 cells. Cells were treated with non-toxic doses of ERα- and ERβ-selective agonist and antagonist for 24 hrs prior to infection. The antagonist treatments were given either alone or were co-administered with 10 nM E2 (protective physiological dose). The MTT tests were performed to determine the cell toxicity of the ERα- and ER-β selective agonist and antagonist at all the doses used for treatment (appendix supplementary Figures 2F-2I).

ERα selective activation by PPT treatment at various doses (0.01 and 0.1 μM) significantly reduced Dr+ *E. coli* invasion in mIMCD3 cells compared to untreated control cells. Maximum protection (32%) against bacterial invasion was observed with 0.1 μM PPT treatment (Figure 14 A). Similar to the treatment with 100 nM E2, increased bacterial invasion was observed in cells treated with a higher dose of PPT (1 μM) compared to invasion in the cells treated with 10 nM E2 and lower doses of PPT. Invasion of Dr+ *E. coli* at 1 μM PPT treatment was not significantly different than that in the untreated control cells. Co-treatment of mIMCD3 cells with ERα-selective antagonist MPP (at doses 0.01, 0.1, and 1 μM) and 10 nM E2 led to significant attenuation of the protective effect of E2 against bacterial invasion in a dose dependent manner (Figure 14 B). Dr+ *E. coli* infected mIMCD3 cells treated with MPP alone at all

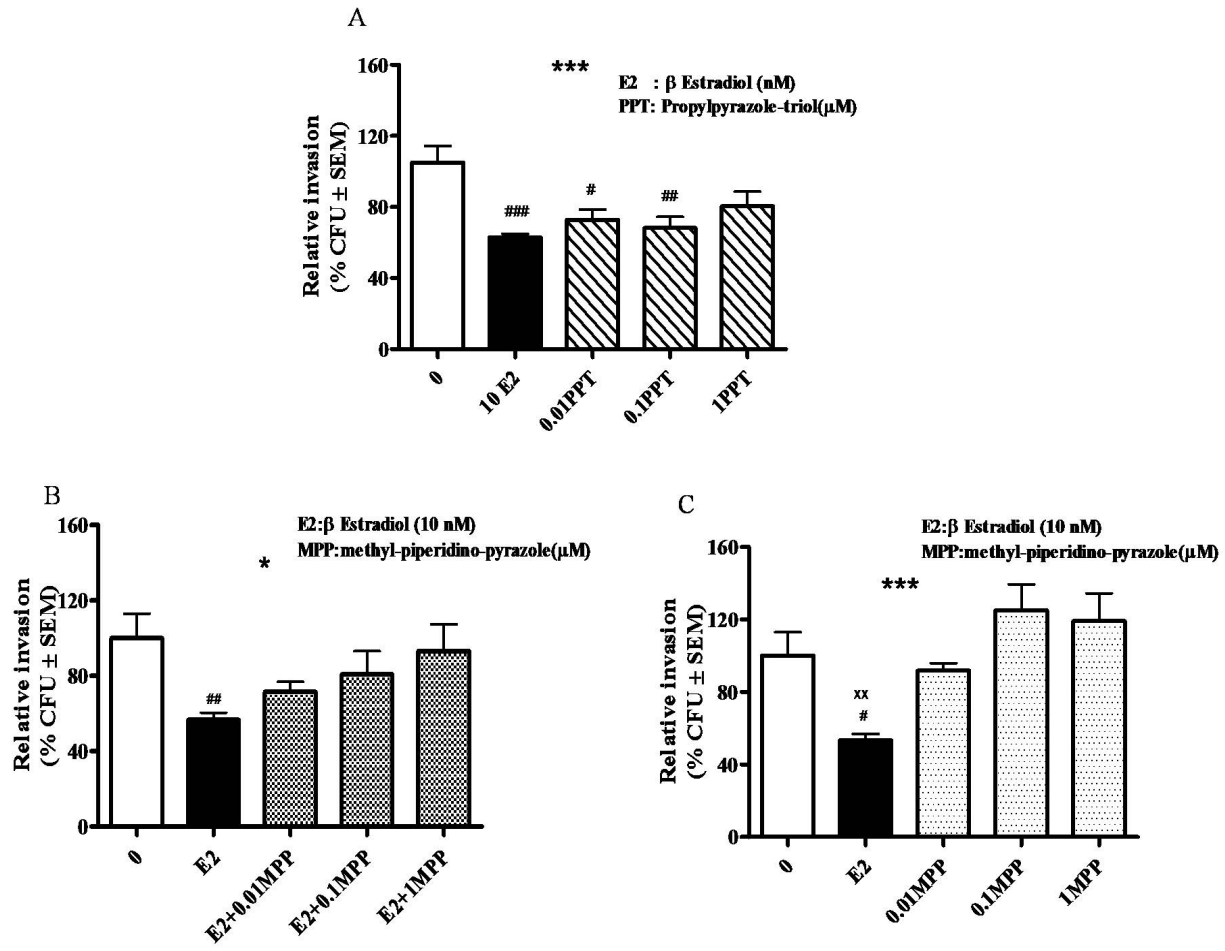


**Figure 14**: **Relative invasion of Dr+ *E. coli* in PPT- and MPP-treated cells.** Relative bacterial invasion following 2 hrs of Dr+ *E. coli* infection in **A)** an ERα-selective agonist, PPT-treated, **B)** E2 and an ERα antagonist MPP-co-treated, and **C)** MPP-alone treated mIMCD3 cells, as determined by gentamicin protection assay. Invasion values are mean bacterial CFU ± SEM expressed as % invasion in treated cells relative to untreated cells (considered as 100 %). The error bars represent data from 4 independent experiments performed in triplicate. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons where $P<0.05$ was considered significant. The character * indicates significant differences among the treatment groups, # indicates significant difference relative to control treatment, x indicates significant difference relative to MPP alone treatment. (Physiological E2 dose1-10 nM)

of the above defined doses showed invasion comparable to the invasion in the untreated control cells, and significantly higher invasion than invasion in the cells treated with 10 nM E2 (Figure 14 C).

In contrast to the ERα- selective agonist PPT, pretreatment of cells with the ERβ selective agonist DPN at various doses (0.01, 0.1 and 1 μM) did not cause significant change in Dr+ *E. coli* invasion compared to the untreated control cells (Figure 15A). The bacterial invasion in DPN treated cells was comparable to that in the untreated controls and was significantly higher than invasion in the 10 nM E2-treated cells (Figure 15A). Furthermore, no reversal of E2-protection against bacterial invasion was observed when the cells were co-treated with 10 nM E2 and the ERβ-selective antagonist R, R-THC at doses 0.1 and 1 μM. Interestingly, a significant increase in the bacterial invasion was observed in the cells co-treated with 10 nM E2 and 10 μM R, R THC (Figure 15B). Treatment with R, R-THC alone at doses of 0.01 and 0.1 μM did not cause significant change in the bacterial invasion when compared to that in the untreated cells; but when cells were treated with at a very high dose of R, R-THC (10 μM), a significant increase in the bacterial invasion was observed (Figure 15C). Notably, there was relatively less bacterial invasion in the cells co-treated with 10 μM R, R-THC and 10 nM E2 compared to the invasion in the cells treated with only 10 μM R, R-THC. This observation suggests that R, R-THC at 10 μM increases bacterial internalization in the cells that appears to be partly reversed by 10 nM E2 treatment (Figure 15 B and C).

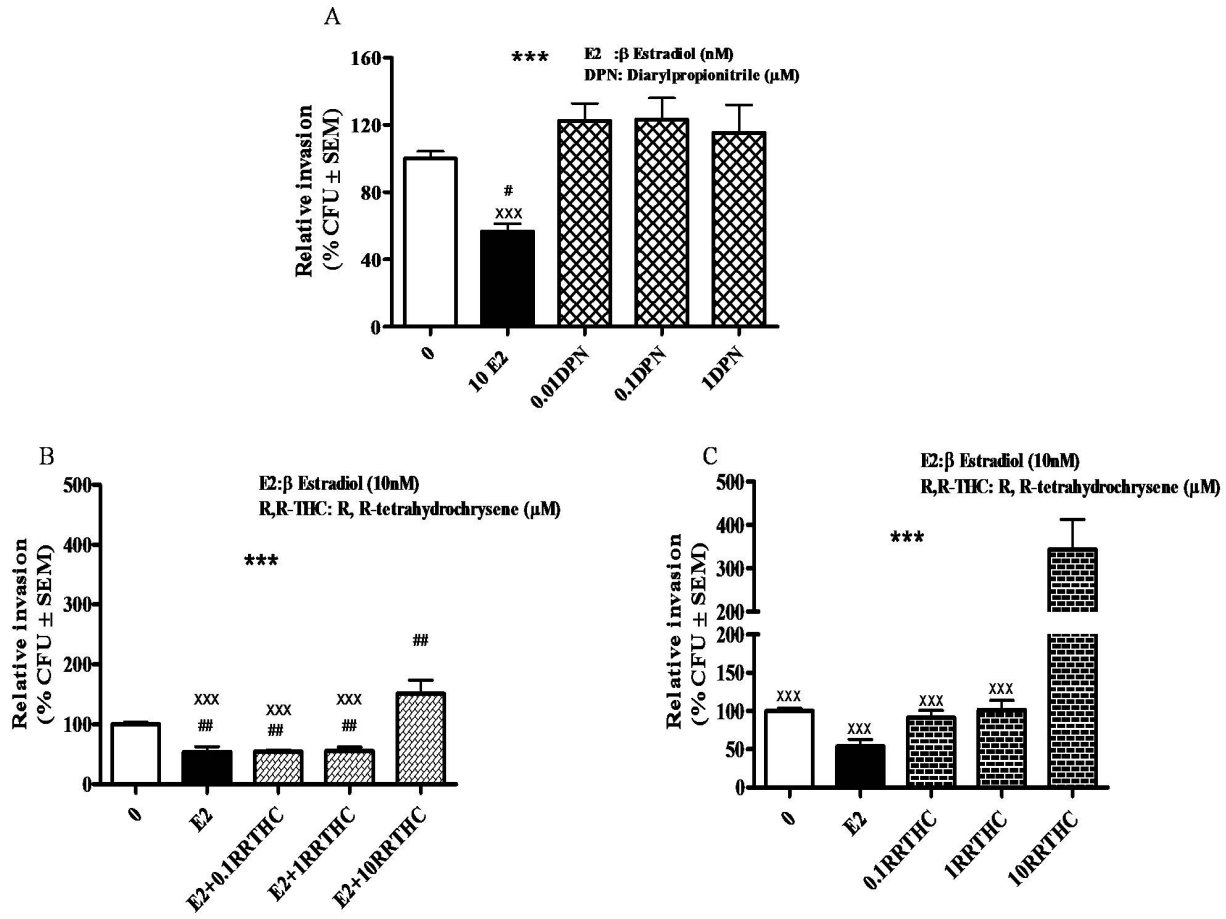


**Figure 15**: **Relative invasion of Dr+ *E. coli* in DPN- and R, R THC-treated cells.** Relative bacterial invasion following 2 hrs of Dr+ *E. coli* infection in **A)** an ERβ-selective agonist, DPN treated, **B)** E2 and an ERβ-antagonist R, R THC co-treated, and **C)** R, R THC-alone treated mIMCD3 cells, as determined by gentamicin protection assay. Invasion values are mean bacterial CFU ± SEM expressed as % invasion in treated cells relative to untreated cells (considered as 100%). The error bars represent data from 4 independent experiments performed in triplicate. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons where $P<0.05$ was considered significant. The character * indicates significant differences among the treatment groups, # indicates significant difference relative to control treatment, and x indicates significant difference relative to R, R THC treatment. (Physiological E2 dose10 nM)

## Discussion

To our knowledge, invasion of Dr+ *E. coli* in mIMCD3 cells and hIMCD cells has not been previously reported. In this study, we have demonstrated for the first time the invasion of pyelonephritic Dr+ *E. coli* strain *in vitro* in mIMCD3 and hIMCD cells. Our study also provides the first *in vitro* evidence for E2/ERα-mediated modulation of Dr+ *E. coli* infection and invasion in mIMCD3 cells. In the present study, E2 treatment at different doses resulted in a significant modulation of Dr+ *E. coli* invasion in mIMCD3 cells in a dose-dependent manner. Kaul *et al* have previously reported significant differences in binding of the Dr adhesins on clinical endometrial samples from different stages of the menstrual cycle (Kaul *et al*, 1996). Furthermore, the ability of vaginal mucus to bind to *E. coli* expressing type1pili has been reported to vary through the menstrual cycle (Venegas *et al*, 1995) .We have observed a dichotomy in effects of estrogen on the Dr+ *E. coli* invasion in mIMCD3 cells at lower- and higher-E2 doses. E2 at physiological doses (1-10 nM) provided marked protection to mIMCD3 and hIMCD cells against Dr+ *E. coli* mediated invasion *in vitro* compared to untreated control cells. In contrast, a pharmacological E2 (100 nM) dose contributed to a significant increase in bacterial invasion in mIMCD3 cells compared to cells treated with a physiological dose of E2. We have also observed E2-mediated protective effects against Dr+ *E. coli* invasion at physiological doses in Huh7 and human hepatocytes. This suggests that the protective effect of E2 at physiological doses against Dr+ *E.coli* invasion is not limited to IMCD3 cells and that various cell types may employ the same molecular mechanisms of Dr+ *E. coli* internalization.

Our *in vitro* results are consistent with our *in vivo* observations of E2-mediated modulation of Dr+ *E. coli* infection in the the mouse urinary tract (results discussed in chapter IV). We have demonstrated significant reductions in colonization of Dr+ *E. coli* in the kidneys of Non-OVX mice, as well as in OVX mice treated with a physiological dose of E2, whereas the estrogen deficient mice showed a marked increase in bacterial colonization. Furthermore, Curran *et al* in their study have demonstrated increased Dr+ *E. coli* colonization in the kidney of OVX mice treated with a pharmacological dose of E2 (Curran *et al*, 2007). Nonetheless, Kaul *et al* have also shown increased susceptibility to Dr+ *E. coli* infection and associated pre-term labor in pregnant C3H/HeJ mice (Kaul *et al*, 1999). Taken together from these observations, it is clearly evident that estrogen at physiological doses provide protection against Dr+ *E. coli* colonization as well as invasion, in contrast, both estrogen deficiency and a pharmacological estrogen dose contribute to increased Dr+ *E. coli* colonization in the mouse urinary tract.

We further report that E2-mediated protection against Dr+ *E. coli* invasion in mIMCD3 cells following infection involves classical activation of ERs. Results from our invasion experiments using ER subtype-specific agonist indicate that ERα plays the predominant role in inducing the protective effect of E2 against Dr+ *E. coli* invasion in mIMCD3 cells. Similar to the effects of E2 administration, treatment with the ERα selective agonist PPT resulted in a significant reduction in Dr+ *E. coli* invasion, whereas treatment with the ERβ selective agonist, DPN did not provide any protection. Similarly, as observed with 100 nM E2 treatment, ERα-selective agonist PPT treatment at a higher dose resulted in relatively elevated bacterial invasion in the cells. Furthermore, ERα

selective antagonist MPP reversed the protective effects of E2; however treatment with ERβ antagonist R, R- THC had no such effects. Our results clearly imply that E2-mediated modulation of Dr+ *E. coli* invasion in mIMCD3 cells is dependent on ERα-activation.

Expression of both, ERα and ERβ were detected in mIMCD3 cells and in the kidneys of C3H/HeJ and B6.129 mice, however only ERα was observed to be functional in determining the E2-mediated protection against Dr+ *E. coli* infection *in vitro.* Our *in vivo* results also demonstrate a functional role of ERα in mediating the protective effects of estrogen against Dr+ *E. coli* colonization in the mouse kidney. We have not investigated the Dr+ *E. coli* colonization in the urinary tracts of ERβ gene knock-out mice in this sudy; however, our *in vitro* results clearly demonstrate that ERβ may not be involved in the protective effects of estrogen against Dr+ *E. coli* infection in the renal cells. Moreover, ERβ mRNA levels were found to be markedly lower than the levels of ERα mRNA in the mouse kidney, as well as in mIMCD3 cells. This may explain the specific role of ERα in mediating E2-effects *in vivo,* as well as *in vitro*. Nonetheless, several studies have reported ERα to be the functional subtype in the mouse kidney (Carley *et al*, 2003, Esqueda *et al*, 2007, Jelinsky *et al*, 2003, Rogers *et al*, 2007).

Binding of UPEC adhesins to the receptors expressed on the host cellular surfaces enables UPEC to colonize and invade the uroepithelium within the urinary tract (Mulvey, 2002). Dr+ *E. coli* adhere to the host uroepithelium by binding to GPI- DAF through its Dr adhesins thus allowing its internalization into the host's uroepithelial cells.

Internalization is regulated via intracellular cytoskeleton rearrangements induced by tyrosine kinase-activated PI3K/Akt signaling (Goluszko *et al*, 1997, Goluszko *et al*, 1999, Goluszko *et al*, 2001, Guignot *et al*, 2001, Korotkova *et al*, 2008, Selvarangan *et al*, 2000). One mechanism by which estrogen modulates Dr+ *E. coli* colonization and invasion in renal cells may involve dose-dependent modification of DAF expression on the renal tubular epithelium. Differential effects of E2 on Dr+ *E. coli* infection and invasion in mIMCD3 cells, as observed in our *in vitro* study, indicate that estrogen via ERα may differentially regulate the expression of colonization receptor DAF in mIMCD3 cells, thereby modulating Dr+ *E. coli* infection and invasion in these cells. Hormonal regulation of DAF expression has been indicated by several studies and regulatory regions of DAF do contain ERE (Kaul *et al*, 1995, Kaul *et al*, 1996, Mirkin *et al*, 2005, Song *et al*, 1996). A second possibility related to the estrogenic modulation of Dr+ *E. coli* cellular invasion may be related to non-genomic mechanisms for estrogen action. Estrogen via ERα may regulate the tyrosine kinase cellular signaling pathway to influence the process of Dr+ *E. coli* internalization in the renal cells. Estrogen through ERs has been reported to regulate PI3K/Akt signaling under different experimental and clinical settings (Gu *et al*, 2009, Simoncini, 2009, Titolo *et al*, 2008). A third potential reason for reduced bacterial colonization and internalization of Dr+ *E. coli* at physiological E2 levels as opposed to increased colonization and internalization at higher estrogen dose and estrogen deficiency could be related to estrogen-mediated modulation of inflammatory responses following infection. Estrogen has the potential to differentially influence the outcome of immune responses following infection. Low physiological levels of estrogen enhance pro-inflammatory cytokines (TNFα, IL6)

production, while higher levels of estrogen stimulate anti-inflammatory cytokines (IL-10) production (Straub, 2007). Such a dichotomy in the effects of different doses of estrogen may modulate the host immune responses,influence the bacterial clearance, as well as colonization following infection.

Based on our *in vitro* and *in vivo* experimental results it seems that estrogen, particularly via ERα, modulates host susceptibility to UPEC colonization and invasion following UTI induction. Targeted disruption of the processes of adhesion and invasion may provide a novel means to prevent and treat recurrent, relapsing and chronic infections within the urinary tract. Furthermore, our results indicate that ERα agonists may play an important role in determining susceptibility to UTIs and can be developed as possible therapeutic drug target to reduce UPEC adhesion and entry during Dr+ *E. coli* induced UTI pathogenesis. We therefore, investigated the possible molecular mechanisms involved in bringing the modulatory effects of estrogen on Dr+ *E. coli* infection *in vitro* and *in vivo*. We next investigated the role of estrogen and ERα on the expression of colonization receptors DAF, *in vivo*, in the mouse kidney and *in vitro*, in mIMCD3 cells following infection with Dr+ *E. coli.*

# CHAPTER VI

# ESTROGEN REGULATES Dr+ *ESCHERICHIA COLI* COLONIZATION RECEPTORS EXPRESSION IN THE MOUSE KIDNEY AND MOUSE INNER MEDULLARY COLLECTING DUCT (mIMCD3) CELLS VIA ESTROGEN RECEPTOR ACTIVATION

## 6.1 Introduction

Adherence to specific colonization receptors on the host uroepithelium is the most crucial event for UPEC to successfully colonize and establish infection in the harsh environment of the host urinary tract (Mulvey, 2002, Sauer *et al*, 2000, Servin, 2005). Adherence also stimulates UPEC entry into the host epithelial cells (Bower *et al*, 2005). Within the urinary tract, DAF expressed at the apical surfaces of the urpoepithelial cells and type IV collagen present in the epithelial basement membrane serve as the colonization receptors for Dr+ *E. coli* (Nowicki *et al*, 1987, Nowicki *et al*, 2001, Vaisanen-Rhen, 1984, Westerlund *et al*, 1989). Epithelial surfaces of the urethra, bladder, ureter and renal pelvis are rich in DAF expression, and there is abundance of type IV collagen in the renal tubular basement membrane as well as in the renal glomerular Bowman's capsule (Nowicki *et al*, 1988). Binding of Dr adhesin to DAF and type IV collagen is crucial for establishment of Dr+ *E. coli* infection in the urinary tract.

Attachment of Dr+ adhesins to DAF allows binding, colonization, and invasion of Dr+ *E. coli* on the uroepithelium and its adherence to type IV collagen enables the uropathogen to colonize the renal interstitium causing persistent infection and pyelonephritis (Nowicki *et al*, 2001, Selvarangan *et al*, 2000, Selvarangan *et al*, 2004).

Our *in vivo* results discussed in chapter IV, indicate that estrogen at a physiological dose provides protection against Dr+ *E. coli* colonization in the mouse urinary tract via the ERα activation pathway, whereas estrogen deficiency and absence of functional ERα increases the colonization of Dr+ *E. coli*. Moreover, increased Dr+ *E. coli* colonization has been observed in the kidneys of OVX mice treated with a high dose of estrogen (Curran *et al*, 2007). This dichotomy of estrogen effects was also observed in our *in vitro* invasion studies using mIMCD3 cells. As discussed in chapter V, significantly lower Dr+ *E. coli* internalization is observed in cells treated with physiological doses of E2 compared to the untreated cells. In contrast, treatment with a pharmacological dose of E2 markedly increases the Dr+ *E. coli* internalization. Furthermore, we found that protective effects of physiological doses of E2 against Dr+ *E. coli* internalization in mIMCD3 cells are mediated primarily by ERα activation.

Adherence of the UPEC in the urinary tract and thus susceptibility to UTIs is primarily determined by the expression pattern of UPEC colonization receptors in the host (Mulvey, 2002). Dr+ *E. coli* adherence to its receptors DAF and type IV collagen has been suggested to be hormonally regulated, and hormonal regulation of DAF and collagen expression has also been reported (Kaul *et al*, 1995, Kaul *et al*, 1996, Lekgabe *et*

*al*, 2006, Song *et al*, 1996). Based on our *in vivo*, as well as *in vitro* studies, we propose that estrogen, via ERα activation, regulates the expression of DAF and type IV collagen and thereby modulates Dr+ *E. coli* colonization and internalization in the the mouse urinary tract, and in mIMCD3 cells. The present study was conducted to determine the effects of estrogen and ERα on the expression of DAF and type IV collagen *in vivo* as well as *in vitro*. We analyzed the expression of DAF and type IV collagen in the kidneys of vehicle- and E2-treated OVX, as well as vehicle- and ICI 182 780-treated Non-OVX, C3H/HeJ mice.

We report here that estrogen deficiency and ER inactivation increase DAF and type IV collagen expression in the kidneys of uninfected as well as Dr+ *E. coli* infected C3H/HeJ mice; whereas E2 replacement at physiological level in OVX mice significantly decreased the expression of these receptors. Furthermore, compared to ERα+/+ mice, ERα-/- mice express higher DAF in the kidneys following infection. Consistent with the *in vivo* results, physiological levels of E2 via ERα, significantly reduced the DAF and type IV collagen expression in mIMCD3 cells; in contrast E2 at a pharmacological dose increased the DAF expression.

## 6.2 Statement of Hypothesis

Estrogen via ERα activation regulates the expression of Dr+ *E. coli* colonization receptors, DAF and type IV collagen, in the host and thus modulates the host's susceptibility to Dr+ *E. coli* induced UTIs.

## 6.3 Results

**A) *In vivo* results**

### 6.3.1: Estrogen deficiency increases the expression of DAF and type IV collagen in the kidneys of OVX C3H/HeJ mice

We analyzed the mRNA levels of DAF and type IV collagen in the kidneys of uninfected, estrogen-deficient OVX, and estrogen–sufficient Non-OVX mice to determine their susceptibility to Dr+ *E. coli* infection. We next investigated the effect of estrogen deficiency on the expression of DAF and type IV collagen in the kidneys of age matched OVX mice at 2, 6 and 21 days post-UTI-induction. Non-OVX mice were used as estrogen-sufficient controls at each of the time points.

Prior to the induction of UTI, the kidneys of estrogen-deficient OVX mice showed significantly higher levels of DAF mRNA compared to the DAF mRNA levels in the kidneys of Non-OVX mice (Figure 16A). At 2 and 6 days post-infection, DAF mRNA levels in kidneys of both, the OVX and the Non-OVX mice were found to be profoundly

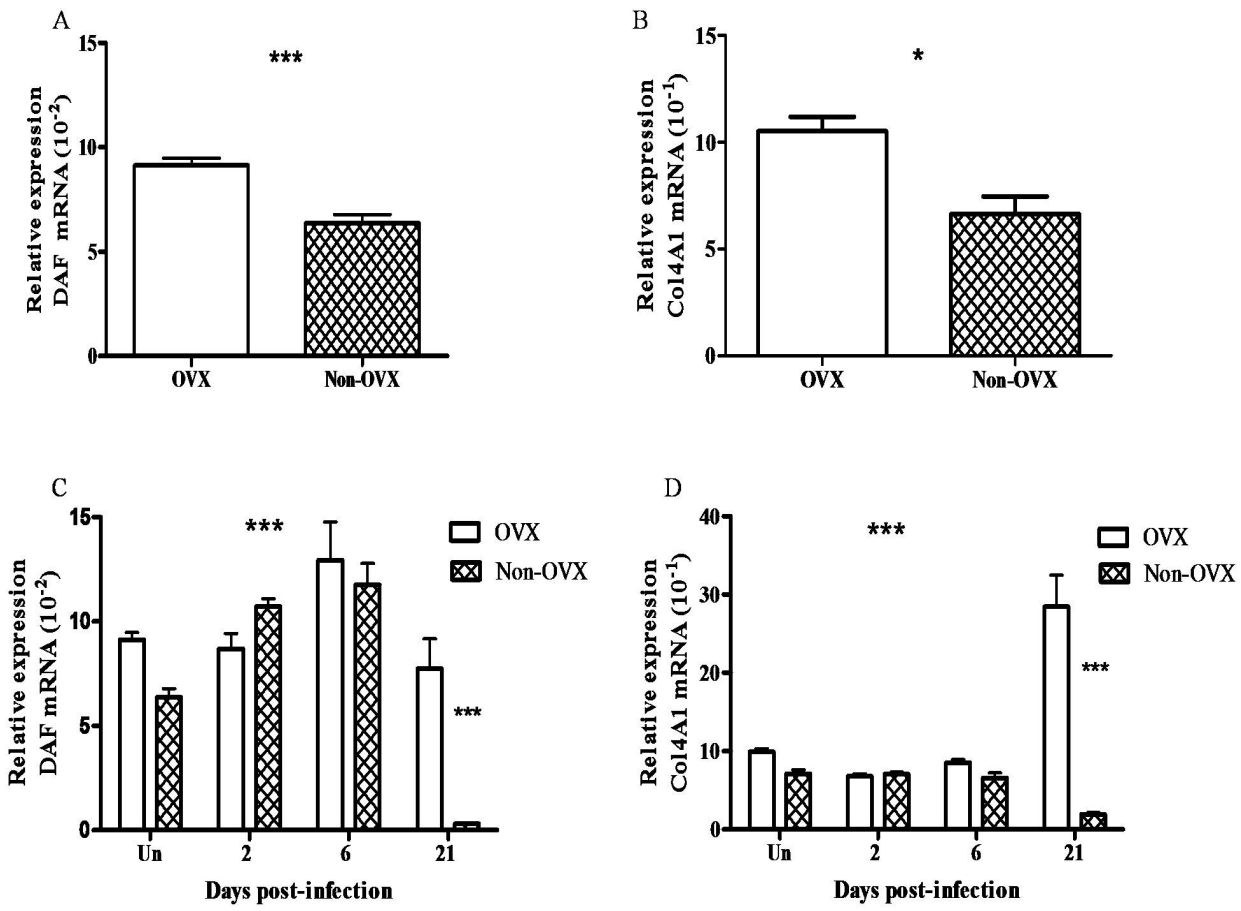


**Figure 16: DAF and type IV collagen subtype A1 (Col4A1) mRNA levels in the kidneys of C3H/HeJ mice**. **A)** The DAF mRNA levels and **B)** the Col4a1 mRNA levels in the kidneys of OVX and Non-OVX mice prior to UTI-induction **C)** DAF mRNA levels and **D)** Col4A1 mRNA levels in the kidneys of OVX and Non-OVX mice at 2, 6 and 21 days post-Dr+ *E. coli* mediated UTI induction. The DAF and Col4a1 mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Target mRNA levels were normalized relative to PPIA mRNA levels in each sample. Expression values are mean relative expression ± SEM and the error bars represent data from 5-8 animals in each study group. Data were analyzed by Student's t- test for Figure 16A and 16B and by Two-Way ANOVA for Figure 16C and 16D where $P< 0.05$ was considered significant. The character, * indicate significant differences among the treatment groups and the character # indicate significant difference in between OVX and Non-OVX mice at 21 day post infection in Figures 16C and 16D

increased compared to DAF mRNA levels in the kidneys of their respective uninfected control mice. However, at 21 days post-infection, DAF mRNA levels declined in the kidneys of OVX as well as Non-OVX mice (Figure 16C).

Among the infected group of mice at 2 days post-infection, DAF mRNA levels in the OVX mice kidneys were lower compared to the levels in the Non-OVX mice kidneys. However, at 6 days post-infection DAF mRNA levels in the OVX mice were higher than those in the Non-OVX mice. These differences were not fornd to be statistically significant. At 21 days post-infection, DAF mRNA levels in the OVX mice kidneys were found to be significantly increased compared to that in the Non-OVX mice kidneys (Figure 16 C). Notably, DAF mRNA levels in the Non-OVX mice at 21 days post-UTI induction were found to be significantly reduced compared to their levels in the uninfected mice and in the mice infected for 2 and 6 days.

Similar to DAF mRNA, levels of type IV collagen mRNA in the kidneys prior to infection were significantly higher in the OVX mice compared to those in the Non-OVX mice (Figure 16B). At 2 and 6 days post-UTI induction, no significant change in type IV collagen mRNA levels was observed in the OVX or the Non-OVX mice compared to those in the uninfected mice. However, at 21 days post-UTI induction, expression of type IV collagen increased significantly in the OVX mice, whereas its expression was markedly reduced in the Non-OVX mice (Figure 16D).

Unlike DAF, at 2 days following infection, the mRNA levels of type IV collagen in the OVX and the Non-OVX mice were not significantly different. However, similar to the observation for DAF, at 6 days post-infection, the mRNA levels of type IV collagen in the OVX mice were found to be higher compared to their levels in the Non-OVX mice. However the differences were not statistically significant. At 21 days post-infection, type IV collagen mRNA levels in the OVX mice was found to be significantly higher compared to those in the Non-OVX mice (Figure 16 D).

### 6.3.2: Estrogen pretreatment decreases expression of DAF and type IV collagen in the kidneys of OVX C3H/HeJ mice post-infection

We analyzed the mRNA levels for DAF and type IV collagen in the kidneys of vehicle- and E2-treated OVX mice, at 21 days post-UTI induction (Figure 17). We also determined the expression of DAF and type IV collagen proteins in the kidneys of these mice (appendix supplementary Figure 3A and 3B).

DAF mRNA levels in the E2-treated mice was significantly lower than in the vehicle-treated mice (Figure 17A). DAF expression was also found to be lower in the E2-treated mice compared to the vehicle-treated mice at the protein level (appendix supplementary Figure 3). Similar to DAF, expression of type IV collagen was also found to be significantly reduced at mRNA (Figure 17B) and protein levels (appendix supplementary Figure 3) in the E2-treated mice compared to the vehicle-treated mice.

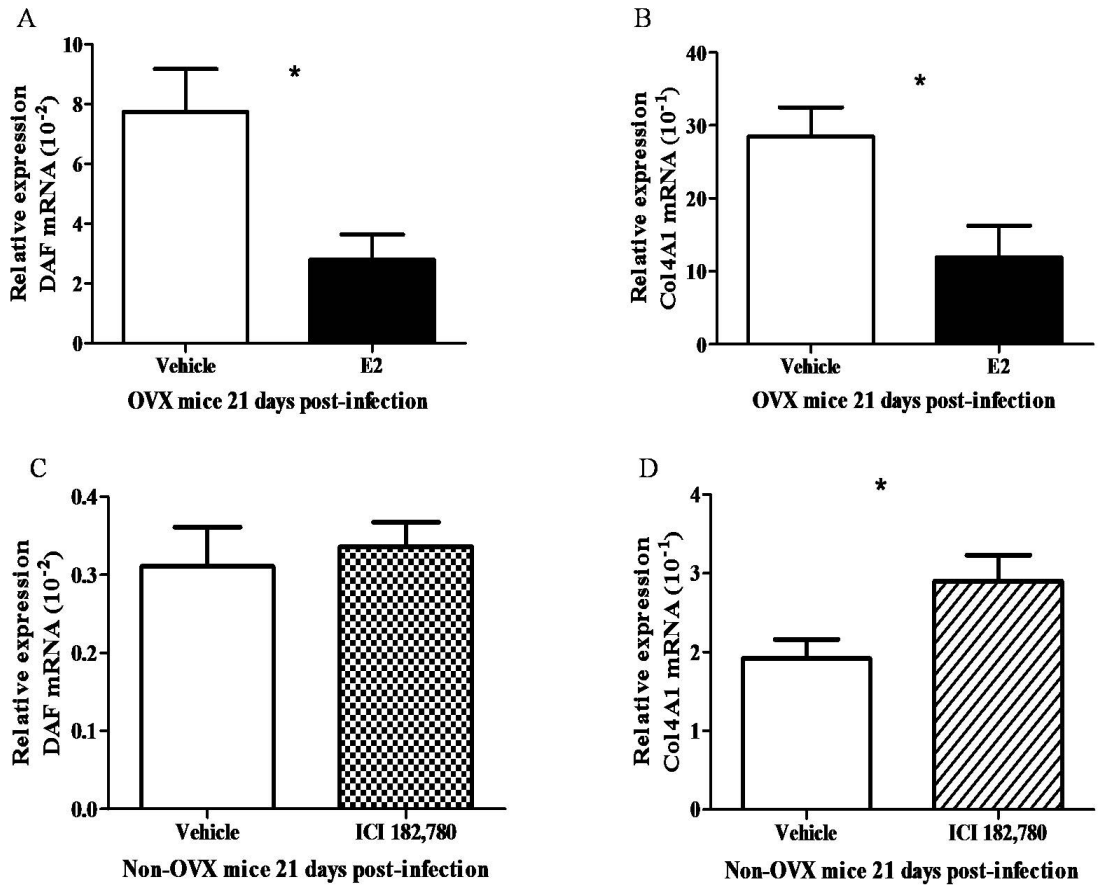


**Figure 17: DAF and Col4A1mRNA levels in the kidneys of E2-treated OVX and ICI 182, 780-treated Non-OVX C3H/HeJ mice**. OVX mice were treated with a physiological dose of E2 and Non-OVX mice were treated with ER complete antagonist ICI 182, 780, prior to induction of infection. **A)** DAF mRNA levels and **B)** Col4a1 mRNA levels in the kidneys of vehicle- and E2-treated OVX and Non-OVX mice at 21 days post-UTI induction. **C)** DAF mRNA levels and **D)** Col4A1 mRNA levels in the kidneys of vehicle- and ICI 182,780-treated Non-OVX mice at 21 days post-UTI induction. The DAF and Col4a1 mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Target mRNA levels were normalized relative to PPIA mRNA levels in each sample. Expression values mean relative expression ± SEM and the error bars represent data from 5-8 animals in each study group. Data were analyzed by Student's t-test where $P<0.05$ was considered significant (*).

### 6.3.3: ER antagonist pretreatment increases DAF and type IV collagen expression in the kidneys of C3H/HeJ mice post-infection

To determine the ER involvement in estrogen-mediated regulation of DAF and type IV collagen expression, we blocked the ERs in the Non-OVX mice with a pure ER-antagonist ICI, 182,780, prior to the induction of experimental UTI. DAF and type IV collagen mRNA levels of in the kidneys of vehicle- and ICI 182, 780-treated mice were determined at 21 days post-UTI induction. Treatment with ER-antagonist resulted in significant increase in DAF and type IV collagen mRNA levels in the kidneys of Non-OVX mice compared to those in the vehicle-treated control mice (Figure 17C and 17D).

### 6.3.4: ERα-/- mice show increased DAF expression in the kidneys compared to the DAF expression in ERα+/+ mice post-infection

To discern the specific role of ERα in transcriptional regulation of DAF and type IV collagen during Dr+ *E. coli* infection, we employed the ERα-/- and ERα+/+ B6.129 female mice. DAF and type IV collagen mRNA levels were measured in the kidneys at 3 and 7 days post-UTI induction.

Figure 18A and 18B represent mRNA levels of DAF and type IV collagen in the kidneys of ERα-/- and ERα+/+ mice. At 3 days post-UTI induction, DAF mRNA levels in ERα-/- and ERα+/+ mice were not significantly different. However, at 7 days post-

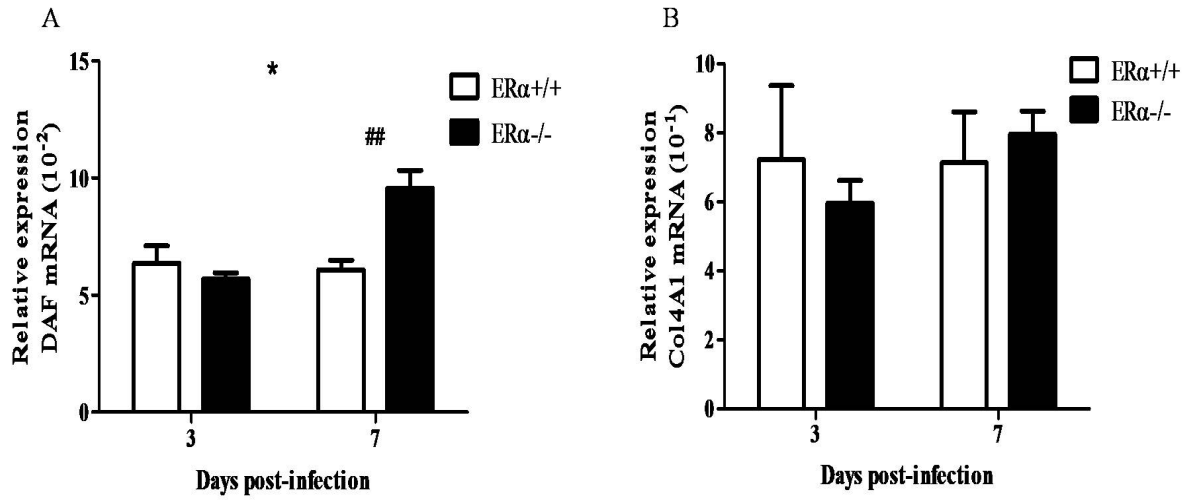


**Figure 18: DAF and Col4A1 mRNA levels in the kidneys of ERα-/- and ERα+/+ mice**. **A)** DAF mRNA levels and **B)** Col4a1 mRNA levels in the kidneys of ERα-/- and ERα+/+ mice at 3 and 7 days post-infection. The DAF and Col4a1 mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Target mRNA levels were normalized relative to PPIA mRNA levels in each sample. Each graph represents expression values from 1 experiment for 3 day infection and 2 experiments for 7 days infection. Expression values are mean relative expression ± SEM and the error bar represents data from 4-7 animals in each study group. Data from 3 day infected ERα-/- mice are from 2 animals. Data were analyzed by Student's t- test and Two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. $P<0.05$ was considered significant. The character * indicate significant difference among the treatment groups, and # indicate significant difference between ERα-/- and ERα+/+ mice.

infection the kidneys of ERα-/- mice had markedly elevated levels of DAF mRNA compared to those of ERα+/+ mice (Figure 18A). At 3 days post-infection type IV collagen mRNA levels in ERα-/- mice kidneys were lower compared to those in ERα+/+ mice. However, at 7 days post-infection, we found increase in type IV collagen mRNA levels in the ERα-/- mice kidney compared to those in the ERα+/+ mice, but the differences were not found to be statistically significant (Figure 18B).

**B) *In vitro* results**

### 6.3.5: E2 regulates expression of DAF and type IV collagen in mIMCD3 cells in a dose-dependent manner

To test if E2-mediated dose-dependent modulation of Dr+ *E. coli* invasion in mIMCD3 cells involves differential regulation of DAF expression, we determined DAF mRNA levels at different doses of E2 in Dr+ *E. coli* infected as well as uninfected mIMCD3 cells. We also analyzed the effect of different E2 doses on type IV collagen expression in mIMCD3 cells. The cells were treated with 0, 1, 10, 50 or 100 nM E2 for 24 hrs (details in chapter III, section 3.3.2) and then were either left uninfected or were infected with Dr+ *E. coli* for 2 hrs. Following infection, cellular levels of DAF and type IV collagen mRNA were determined (details in chapter III section 3.4).

Figure 19A represents the mRNA levels of DAF in mIMCD3 cells treated with various E2 doses. In both uninfected and infected cells, DAF mRNA levels remained

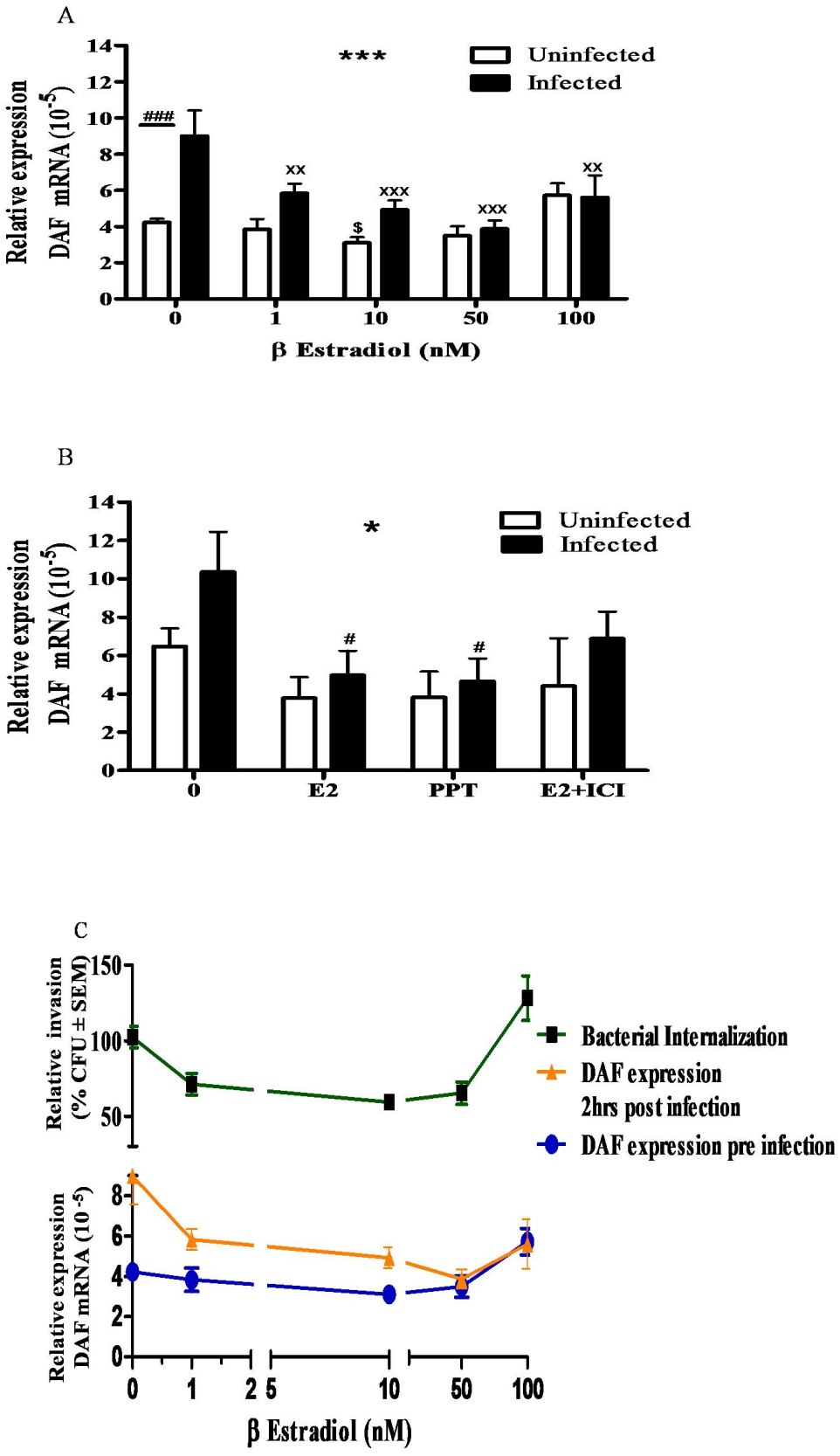
A
Relative expression DAF mRNA (10-5)
Uninfected
Infected
***
###
xx
xxx
$
xxx
xx
0
1
10
50
100
β Estradiol (nM)
B
Relative expression DAF mRNA (10-5)
Uninfected
Infected
*
#
#
0
E2
PPT
E2+ICI
C
Relative invasion (% CFU ± SEM)
Bacterial Internalization
DAF expression 2hrs post infection
DAF expression pre infection
Relative expression DAF mRNA (10-5)
0
1
2
5
10
50
100
β Estradiol (nM)

**Figure 19: DAF mRNA levels in the mIMCD3 cells treated with E2, ICI 182, 780 (ICI) or PPT.** DAF mRNA levels relative to PPIA were analyzed by quantitative SYBR green real-time RT-PCR in Dr+ *E. coli* infected and uninfected mIMCD3 cells treated either with **A**) E2 at different doses or **B)** 10 nM E2, 10 nM E2+10 μM ICI,or 0.1 μM PPT. Untreated cells served as controls. Expression values are mean relative expression ± SEM and the error bars represent data from at least 4 experiments, performed in triplicate. Data were analyzed by Two-way ANOVA followed by Bonferroni post hoc test where $P<0.05$ was considered significant. The character * indicate significant differences among treatment groups, x indicate significant difference relative to control in infected cells, $ indicate significant differences relative to 100 nM E2 treatment in uninfected cells, and # indicate significant differences in between infected and uninfected cells. **C)** DAF mRNA expression and Dr+ *E. coli* relative invasion in mIMCD3 cells treated with different doses of E2. Invasion values are mean bacterial CFU ± SEM expressed as relative % invasion in E2-treated cells relative to untreated cells (invasion in untreated cells is considered to be 100%).

significantly reduced in the cells treated with physiological E2 doses (1-10 nM), compared to those in the untreated cells (Figure 19A). The cells treated with 10 nM E2 expressed significantly lower DAF compared to the cells treated with a higher (100 nM) dose of E2 (Figure 19A). Infection with Dr+ *E. coli* resulted in up-regulation of DAF expression in control as well as E2-treated cells at all E2 doses, except at 100 nM (Figure 19 A). Notably, among the E2-treated cells, the relative increase in DAF expression in the infected cells compared to uninfected cells remained low (Figure 19 A). However, in the control untreated group of mIMCD3 cells, DAF mRNA levels in infected cells are significantly higher compared to those in the uninfected cells (Figure 19A).

Figure 19C represents the bacterial invasion and DAF mRNA levels in E2-treated mIMCD3 cells. The bacterial invasion at physiological doses of E2 (1-10 nM) was lower compared to the bacterial invasion in the untreated control cells and also to the cells treated with a higher dose of E2 (100 nM). Notably, bacterial invasion in cells correlated with the DAF mRNA levels at different E2 doses, where higher DAF mRNA corresponds with increased bacterial invasion and lower DAF mRNA corresponds with reduced bacterial invasion.

Type IV collagen mRNA levels in both infected and uninfected cells were found to be markedly reduced in E2-treated cells at all the doses compared to that in untreated cells (Figure 20A). Infection with Dr+ *E. coli* increased type IV collagen expression in all the treatment groups compared to that in uninfected cells (Figure 20A). Similar to the

observations for DAF expression, the relative increase in type IV collagen mRNA levels following infection in the E2-treated cells remained lower compared to the relative increase in untreated cells (Figure 20A).

### 6.3.6: E2 regulates expression of DAF and type IV collagen in mIMCD3 cells via ERα activation pathway

To test the involvement of ERs in E2-mediated regulation of DAF and type IV collagen expression in mIMCD3 cells, we co-treated the cells with E2 (10 nM) and the ER complete antagonist ICI 182,780 (10 μM). DAF and type IV collagen mRNA levels were analyzed in both Dr+ *E. coli* infected and uninfected cells following treatment with ICI, 182 780.

ER antagonist treatment led to an increase in DAF mRNA levels compared to E2-treated cells, following infection (Figure 19B). Blocking of ERs with ICI 182, 780 resulted in a significant increase in type IV collagen mRNA levels in infected as well as uninfected cells compared to those in the E2-treated cells.

To further determine the role of ERα on estrogen-mediated regulation of DAF and type IV collagen expression, mIMCD3 cells were pretreated with an ERα specific agonist 0.1 μM PPT (at the dose that provided protection against Dr+ *E. coli* invasion in mIMCD3 cells, chapter V section 5.3.3). DAF and type IV collagen mRNA levels were measured in PPT treated Dr+ *E.coli* infected as well as uninfected cells.

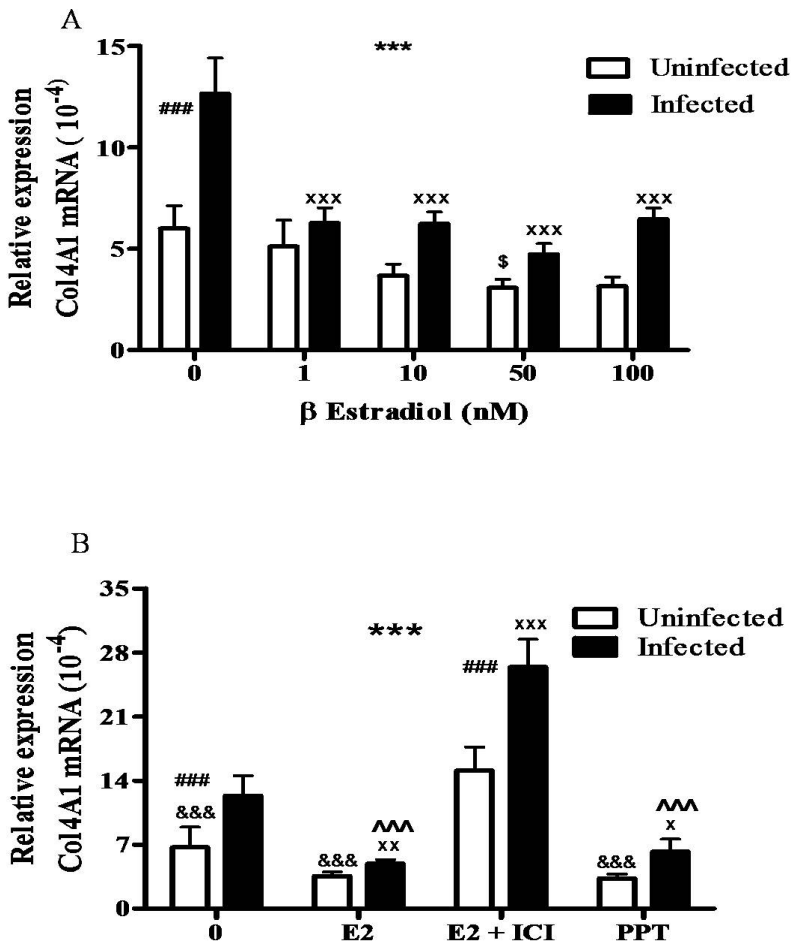


**Figure 20: Col4A1 mRNA levels in the mIMCD3 cells treated with E2, ICI 182, 780 (ICI) or PPT.** Col4A1 mRNA levels relative to PPIA were analyzed by quantitative SYBR green real-time RT-PCR in Dr+ *E. coli* infected and uninfected mIMCD3 cells treated either with **A**) E2 at different doses or **B)** 10 nM E2, 10 nM E2+10 μM ICI, 0.1 μM PPT. Untreated cells served as controls. Expression values are mean relative expression ± SEM and the error bars represent data from at least 4 experiments performed in triplicate. Data were analyzed by Two-way ANOVA followed by Bonferroni post hoc test where $P<0.05$ was considered significant. The character * indicates significant differences among treatment groups, x indicates significant differences relative to control in infected cells, $ indicates significant differences relative to control in uninfected cells, ^ indicates significant differences relative to E2+ ICI treatment in infected cells, & indicates significant differences relative to E2+ ICI treatment in uninfected cells, and # indicates significant differences between infected and uninfected cells for each treatment group.treated cells were comparable to their expression in cells treated with E2 at a physiological dose (10 nM).

In both uninfected and infected cells, PPT treatment led to significant decrease in DAF (Figure 19B), as well as type IV collagen (Figure 20B) mRNA levels, compared to the levels in untreated cells. The expression of DAF and type IV collagen in 0.1 μM PPT treated cells were comparable to their expression in the cells treated with E2 at a physiological dose (10nM).

## 6.4 Discussion

An influence of estrogen on the adherence of UPEC to female urogenital tract has been suggested by several studies (Schaeffer *et al*, 1979, Sharma *et al*, 1987, Sobel & Kaye, 1986). However, the precise molecular mechanism associated with the estrogenic effect on UPEC adhesion has not been previously described. In this study, we demonstrate for the first time that estrogen, via ERα, regulates colonization and internalization of Dr+ *E. coli* by regulating the expression of its colonization receptors, DAF and type IV collagen, *in vivo* in the mouse kidney and *in vitro,* in mIMCD3 cells. Our study also provides the first direct *in vitro* evidence for estrogen-mediated dose-dependent regulation of DAF and type IV collagen expression in mIMCD3 cells during Dr+ *E. coli* infection.

Prior to infection, estrogen deficient menopausal OVX C3H/HeJ mice expressed significantly higher levels of DAF and type IV collagen in the kidneys compared to those in estrogen-sufficient Non-OVX mice, suggesting increased susceptibility of OVX mice

to Dr+ *E. coli* colonization. Following infection, the OVX mice showed higher DAF as well as type IV collagen expression compared to the Non-OVX and E2-treated OVX mice. Consistent with these observations we have found increased Dr+ *E. coli* colonization in OVX mice compared to that in Non-OVX and OVX mice treated with physiological doses of E2 (Discussed in chapter IV). Binding of Dr+ *E. coli* to DAF and type IV collagen is essential for bacterial colonization and initiation of infection in the renal tissues (Nowicki *et al*, 2001, Selvarangan *et al*, 2000, Selvarangan *et al*, 2004). Estrogen-mediated reduction in DAF expression may attenuate the adherence of Dr+ *E. coli* to the host uroepithelium. Similarly, reduction in type IV collagen expression will lower type IV collagen deposition in the epithelial basement membrane and thus reduce susceptibility to Dr+ *E. coli* colonization in the renal interstitium. This will result in reduced susceptibility to Dr+ *E. coli* colonization and inflammation-induced kidney fibrosis in the host following infection. Taken together, results from this study support our hypothesis and provide evidence for a possible molecular mechanism associated with the protective effect of host physiological estrogen levels against Dr+ *E. coli* induced UTI and increased susceptibility to Dr+ *E. coli* infection in estrogen deficient conditions.

Furthermore, our *in vitro* studies utilizing mIMCD3 cells show dose-dependent regulation of DAF expression by E2 that correlates with the dose-dependent modulation of Dr+ *E. coli* invasion in the cells (Figure 19C and results discussed in chapter V). Treatment of cells with physiological doses of E2 resulted in attenuated DAF expression and a 40-60% reduction in Dr+ *E. coli* invasion compared to that in untreated cells. Thus, it is clear that physiological E2 levels reduce DAF expression resulting in reduction

of bacterial attachment and invasion; whereas expression of DAF is increased in cells growing under E2 deficient conditions, resulting in higher bacterial invasion.

Significant increases in DAF expression and Dr+ *E. coli* internalization were also observed in mIMCD3 cells treated with a pharmacological dose of E2. High estrogen levels during pregnancy have been implicated in increased risk for cystitis and pyelonephritis which in turn may contribute to premature delivery and congenital anomalies (Kaul *et al*, 1999, Mittal & Wing, 2005, Santos *et al*, 2002, Schnarr & Smaill, 2008). Furthermore, Kaul *et al* have reported differential adherence of Dr adhesins to endometrial samples from females with normal menstrual cycle, showing luminal up-regulation of DAF expression during the secretory phase (Kaul *et al*, 1995, Kaul *et al*, 1996). Interestingly, Curran *et al* have also reported increased Dr+ *E. coli* colonization in the kidneys of OVX C3H/HeJ, as well as in the C3H/HeN (LPS responder) mice treated with a higer level of E2 (Curran *et al*, 2007). Importantly, treatment with a higher E2 level in these mice was not found to alter the colonization of Dr adhesin mutant *E. coli* (Dr- *E. coli*) that has lost the capacity to adhere to DAF and invade the host cells (Curran *et al*, 2007). Based on our result, it can be inferred that in the study by Curran *et al*, higher estrogen levels may possibly have led to up-regulation of adhesion receptor DAF expression thus resulting in increased infection by Dr+ *E. coli* but not by Dr- *E. coli*.

Studies investigating estrogen-mediated gene regulation have revealed that there is significant diversity among estrogen response networks found in different cells and at different doses. Variation in expression networks can occur due to the occupancy of

different ERα/ ERβ binding sites in the genome, and may also be determined by the co-factor compositions of a particular cell type (McDonnell *et al*, 1995, Nilsson *et al*, 2001, Nilsson & Gustafsson, 2002). From our results, it is now evident that host's endogenous estrogen levels play an important role in determining host susceptibility to Dr+ *E. coli* induced UTI by regulating DAF and type IV collagen expression in the renal tissue and cells. Kaul *et al* have also shown regulation of DAF expression by progesterone. However in this study, we have not investigated the effects of progesterone on DAF or type IV collagen expression (Kaul *et al*, 1995).

Utilizing ERα-/-mouse model and ERα specific agonist PPT treatment in mIMCD3 cells, we demonstrate for the first time that estrogen-mediated regulation of DAF and type IV collagen in the mouse kidney and mIMCD3 cells is ERα dependent. Expression of DAF and type IV collagen has been previously reported to be regulated by estrogen (Potier *et al*, 2001, Potier *et al*, 2002, Song *et al*, 1996). Estrogen-mediated regulation of DAF expression has earlier been reported in the mouse uterus (Song *et al*, 1996). Regulatory regions of the DAF gene do contain ERE and induction of mouse DAF by estrogen has been found to be tissue specific (Mirkin *et al*, 2005, Song *et al*, 1996). Besides ERE, DAF gene regulatory regions also contain Sp1, Ap1and NFκB transcription factor binding sites and regulation of its expression has been reported to include ER mediated transcriptional and cellular signaling activation pathways, MAPK and NFκB (Ahmad *et al*, 2003, Cauvi *et al*, 2006, Mirkin *et al*, 2005, O'Brien *et al*, 2008, Thomas & Lublin, 1993). E2 is known to regulate activation of MAPK pathway and there seems to exist a cross-talk between MAPK and ER subtype activation (Altiok *et al*,

2007, Endoh *et al*, 1997, Frigo *et al*, 2006, Lee & Bai, 2002, Lee & Eghbali-Webb, 1998, Migliaccio *et al*, 1996, Ruzycky, 1996, Seval *et al*, 2006, Wade & Dorsa, 2003). Estrogen-mediated regulation of collagen type IV expression has been found to be renoprotective because estrogen-mediated down regulation of type IV collagen expression following injury or infection attenuates renal fibrosis (Neugarten *et al*, 2000, Potier *et al*, 2002). The promoter of type IV collagen has not yet been reported to contain ERE, and E2-mediated transcriptional regulation of type IV collagen occurs via cellular signaling pathway activated Sp1 transcription factor (Burbelo *et al*, 1988, Zdunek *et al*, 2001). In the kidney, E2 is reported to suppress collagen type IV expression by downregulating TGFβ and TGFβ receptors (Dixon & Maric, 2007, Milanini-Mongiat *et al*, 2002, Potier *et al*, 2002). MAPK has been reported to regulate DAF and collagen type IV expression (Ahmad *et al*, 2003, Dixon & Maric, 2007, Milanini-Mongiat *et al*, 2002, O'Brien *et al*, 2008, Potier *et al*, 2002). Using Huh-7 cells, we have previously shown that E2-mediated protection against Dr+ *E. coli* invasion involves MAPK activation (Kaul *et al*, 2007), however we have not yet investigated the interaction of ERs and MAPK in the regulation of DAF and type IV collagen expression in mIMCD3 cells or *in vivo*.

In our study, we found that infection increased DAF as well as type IV collagen expression, both *in vivo* and *in vitro*. Notably, in the *in vitro* study we found that the relative increases in DAF and type IV collagen expression following infection in E2-treated cells were lower than the relative increase observed in the untreated control cells. Consistent with this, our *in vivo* results also indicate that following infection the relative

increase in DAF and type IV collagen expression is higher in the estrogen-deficient OVX mice compared to the estrogen-sufficient Non-OVX and to the E2-treated OVX mice. These observations suggest the existence of additional regulatory mechanisms for DAF and type IV collagen expression following infection that may be associated with influence of estrogen on infection induced inflammatory responses. Both DAF and type IV collagen expression have been found to be up-regulated following activation of inflammatory pathways. Pro-inflammatory cytokines including TNFα and chemokines including IL8 have been reported to up-regulate DAF as well as type IV collagen expression following injury and infection, *in vivo*, as well as *in vitro*(Betis *et al*, 2003b, Fang *et al*, 2004, Mankhey *et al*, 2007, Meldrum *et al*, 2007, Nowicki *et al*, 2009, Potier *et al*, 2002). Moreover, estrogen is known to have immunomodulatory effects on the inflammatory responses showing pro-as well as anti-inflammatory effects (Cutolo *et al*, 2004, Straub, 2007). Thus, the estrogen-mediated regulation of DAF and type IV collagen may involve direct estrogen/ER-mediated regulation of their expression as well as indirect regulation via modulation of inflammatory responses. We test the regulatory effect of estrogen on inflammation following Dr+ *E. coli* infection later in the study (discussed in chapter VIII).

Based on our observations from this study, we conclude that adherence of uropathogens to uroepithelium is a dynamic process and is differentially influenced by estrogen levels. Physiological levels were found to be protective, whereas, both higher and reduced doses of estrogen contribute to increased UTI susceptibility. Furthermore, our study provides the first *in vitro*, as well as *in vivo* evidences, for ERα mediated

modulation of Dr+ *E. coli* colonization and internalization in the host via regulation of its colonization receptors DAF, and type IV collagen expression. Furthermore, we propose that ERα selective ligands may serve as possible therapeutic targets against Dr+ *E. coli* mediated UTI in estrogen deficient and in postmenopausal females.

# CHAPTER VII

# ESTROGEN MODULATES Dr+ *ESCHERICHIA COLI* INVASION IN THE MOUSE INNER MEDULLARY COLLECTING DUCT (mIMCD3) CELLS VIA REGULATING TYROSINE KINASE ACTIVATION PATHWAY

## 7.1 Introduction

UPEC employs diverse mechanisms to invade the host uroepithelium including manipulation of cellular signaling, hijacking of host complement receptors via opsonization, binding to adhesin receptors, and activating cytoskeletal rearrangement (Bower *et al*, 2005, Dhakal & Mulvey, 2009, Li *et al*, 2006, Mulvey, 2002, Palmer *et al*, 1997, Springall *et al*, 2001). Uptake of uropathogenic bacteria by renal epithelial cells (Donnenberg *et al*, 1994, Palmer *et al*, 1997, Straube *et al*, 1992, Warren *et al*, 1988) has been found to be associated with tyrosine phosphorylation of specific host proteins (Palmer *et al*, 1997). Adherence of pyelonephritic Dr+ *E. coli* to its colonization receptor DAF causes recruitment of α5β1integrins and additional DAF at the sites of bacterial attachment on the epithelial cell followed by bacterial internalization (Das *et al*, 2005, Goluszko *et al*, 1999, Guignot *et al*, 2001, Kansau *et al*, 2004, Plancon *et al*, 2003, Selvarangan *et al*, 2000). Engagement of DAF with members of Afa/Dr adhesin bearing *E. coli* has been shown to induce tyrosine kinase mediated PI3K/Akt signaling activation

(Cane *et al*, 2007, Peiffer *et al*, 1998) that triggers bacterial internalization by the host cells (Brumell & Grinstein, 2003, Goluszko *et al*, 1997, Goluszko *et al*, 1999, Goluszko *et al*, 2008, Wymann & Pirola, 1998). Using our *in vitro* experimental UTI model we have demonstrated for the first time that Dr+ *E. coli* is internalized by mIMCD3 cells (results from chapter V). However, involvement of tyrosine kinase activation during Dr+ *E. coli* invasion in mIMCD3 cells has not been previously investigated. In the present study we tested the role of tyrosine kinase in Dr+ *E. coli* internalization in mIMCD3 cells.

Our in *vitro* studies indicate that E2 modulates Dr+ *E. coli* invasion in mIMCD3 cells via regulating DAF expression (chapter VI). Both *in vitro* and *in vivo* studies have reported that estrogen has regulatory effects on the tyrosine kinase activated PI3K/Akt signaling pathway in different cell types and under various physiological conditions *in vitro* as well as *in vivo* (Boonyaratanakornkit & Edwards, 2007, Cato *et al*, 2002, Edwards & Boonyaratanakornkit, 2003, Hammes & Levin, 2007). For example, E2 treatment in MCF-7 cells and in endometrial cancer cells has been shown to increase tyrosine kinase activity and promptly activate the PI3K/Akt signal pathway. In contrast, positive effects of E2 treatment on post ovariectomy osteoporosis have been associated with inhibition of tyrosine kinase activity in osteoclast cells (Guo *et al*, 2006, Li & Yu, 2003, Liu & Howard, 1991, Migliaccio *et al*, 1996, Pascoe & Oursler, 2001, Yoneda *et al*, 1993). In addition, genistein, a compound that has structural similarity to E2 and binds to ERs is a known tyrosine kinase inhibitor; it has been observed to mimic beneficial biological effects of E2 on bone tissue by inhibiting osteoclast activity (Gao &

Yamaguchi, 1999, Gao & Yamaguchi, 2000, Li & Yu, 2003). Based on these observations, we propose that E2-mediated modulation of Dr+ *E. coli* invasion in mIMCD3 cells may also include regulation of tyrosine kinase signaling. Thus, the present study was conducted to investigate the role of tyrosine kinase and PI3K/Akt signaling in Dr+ *E. coli* invasion in mIMCD3 cells and to further test if estrogen modulates tyrosine kinase and PI3K/Akt signaling activation to influence Dr+ *E. coli* invasion in these cells.

In the present study, we found that inhibition of tyrosine kinase in mIMCD3 cells caused significant reductions in Dr+ *E. coli* invasion. We also found that E2 at a physiological dose and at a higher dose differentially regulates PI3K and Akt signal activation resulting in different outcomes on Dr+ *E. coli* invasion in mIMCD3 cells.

## 7.2 Statement of Hypothesis

Dr+ *E. coli* invasion in mIMCD3 cells require tyrosine kinase activation. Furthermore, estrogen regulates tyrosine kinase activation to modulate Dr+ *E. coli* invasion in mIMCD3 cells.

## 7.3 Results

### 7.3.1: Tyrosine kinase activation induces Dr+ *E. coli* invasion in mIMCD3 cells

To determine the role of the tyrosine kinase signaling pathway during Dr+ *E. coli* invasion in mIMCD3 cells, we treated these cells with known tyrosine kinase inhibitors prior to infection. Genistein, a phytoestrogen is an ER agonist with known tyrosine kinase inhibitor activity and LY294002 is a potent PI3K inhibitor. The cells were pretreated with non-toxic doses of genistein (0.01, 0.1, 1 and 10 µM) or LY294002 (10, 50 and 100 µM) or left untreated. Genistein treatment was given for 24 hrs and LY294002 treatment was given for 15 min prior to infection. Following treatment, the cells were either left uninfected or were infected with Dr+ *E. coli* for 2 hrs and bacterial internalization was determined by the gentamicin protection assay. Total infection (adhered and internalized bacteria) in mIMCD3 cells was determined as described in the methods section (chapter III). Drug toxicity for genistein and LY294002 treatment in mIMCD3 cells was tested by MTT assay (appendix supplementary Figures 2D and 2E). The phosphorylated PI3K and Akt (pPI3K and pAkt) protein levels were detected by western blot to evaluate the activation status of tyrosine kinase in genistein and LY294002 treated, Dr+ *E. coli* infected and uninfected mIMCD3 cells.

Pretreatment of the mIMCD3 cells with genistein significantly reduced Dr+ *E. coli* internalization in mIMCD3 cells (Figure 21 A). Importantly, genistein blocked uptake of Dr+ *E. coli* in a dose-dependent manner resulting in more than 60% inhibition at a 10µM dose (Figure 21A). To further address the role of tyrosine kinase-activated PI3K signaling in Dr+ *E. coli* invasion, we inhibited the PI3K signaling pathway in mIMCD3

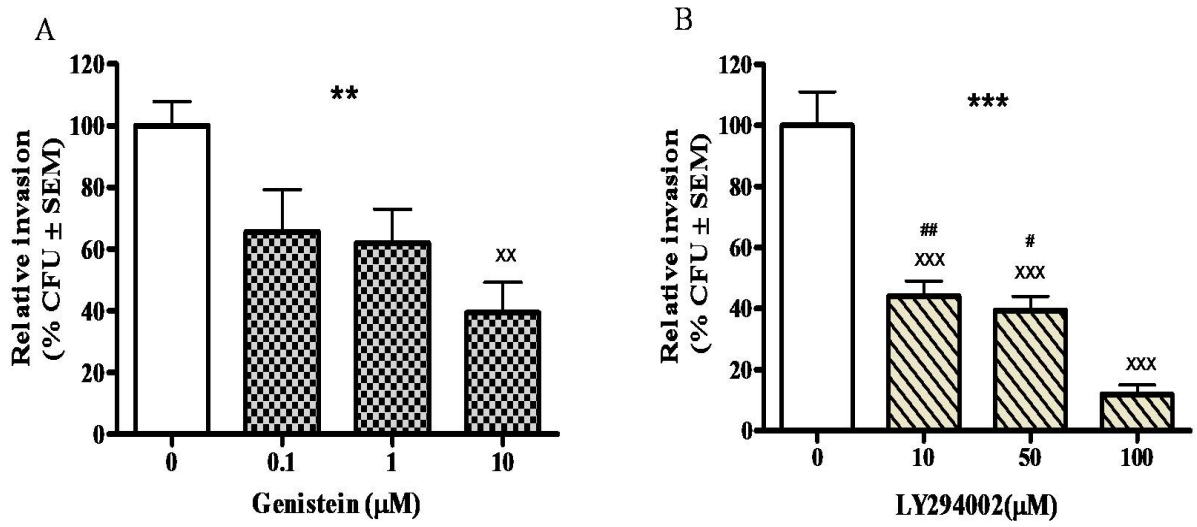


**Figure 21: Relative invasion of Dr+ *E. coli*in the mIMCD3 cells treated with tyrosine kinase inhibitors.** Relative bacterial invasion following 2 hrs of Dr+ *E. coli* infection in mIMCD3 cells treated with **A)** tyrosine kinase inhibitor genistein (0.1, 1 or 10 μM) and **B)** PI3K inhibitor LY294002, as determined by gentamicin protection assay. Invasion values are mean bacterial CFU ± SEM expressed as % invasion in treated cells relative to untreated cells (considered as 100%). The error bars represent data from at least 4 independent experiments performed in triplicate. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons where $P < 0.05$ was considered significant. The character * indicate significant differences among the treatment groups, x indicate significant differences compared to control treatment in Figures 21A and 21B, and # indicate significant differences compared to the 100 μM LY294002 treatment in Figure 21B.

cells by LY294002. Clearly, PI3K inhibition strongly impaired uptake of Dr+ *E. coli* in mIMCD3 cells in a dose-dependent manner resulting in up to 90% reduction in invasion compared to that in the untreated control cells (Figure 21B**)**. No significant differences were found in the levels of total bacterial infection (bacteria adhered plus internalized as determined by the total CFU counts) in genistein and LY294002 treated mIMCD3 cells (Figure 22A-22B). The amount of bacteria internalized was significantly reduced in genistein and LY294002 treated cells (Figures 22 C and 22D) and there was marked reduction in the rate of bacterial internalization (% of bacteria internalized/ bacteria infected) in a dose dependent manner (Figure 22D-F).

To confirm the inhibition of tyrosine kinase activity, we analyzed the protein levels of pPI3K and pAkt in both genistein-(10 μM) and LY294002-(10, 50 and 100 μM) treated uninfected and Dr+ *E. coli* infected mIMCD3 cells (Figure 23 and 24). These inhibitors decreased tyrosine kinase activation, resulting in blocking of cellular bacterial invasion in a dose dependent manner. Notably, LY294002 treated cells showed significant dose dependent decreases in pPI3K and pAkt levels, with maximum reduction at a 100 μM dose. Marked reductions in pPI3K and pAkt levels were found in genistein treated cells compared to the untreated control cells.

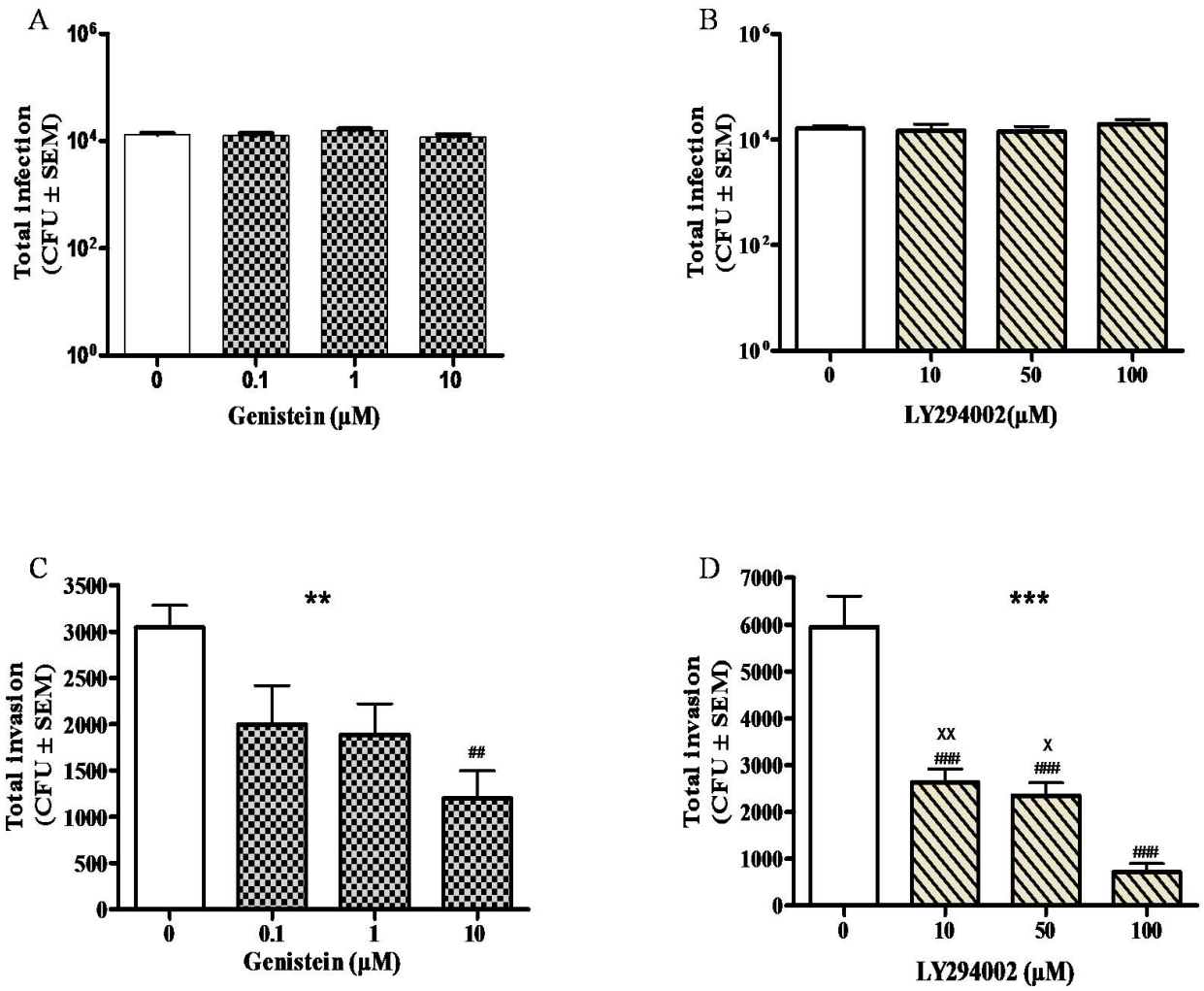
A
Total infection (CFU ± SEM)
Genistein (µM)
B
LY294002(µM)
C
Total invasion (CFU ± SEM)
**
##
Genistein (µM)
D
***
xx
###
x
###
###
LY294002 (µM)

**Figure 22**

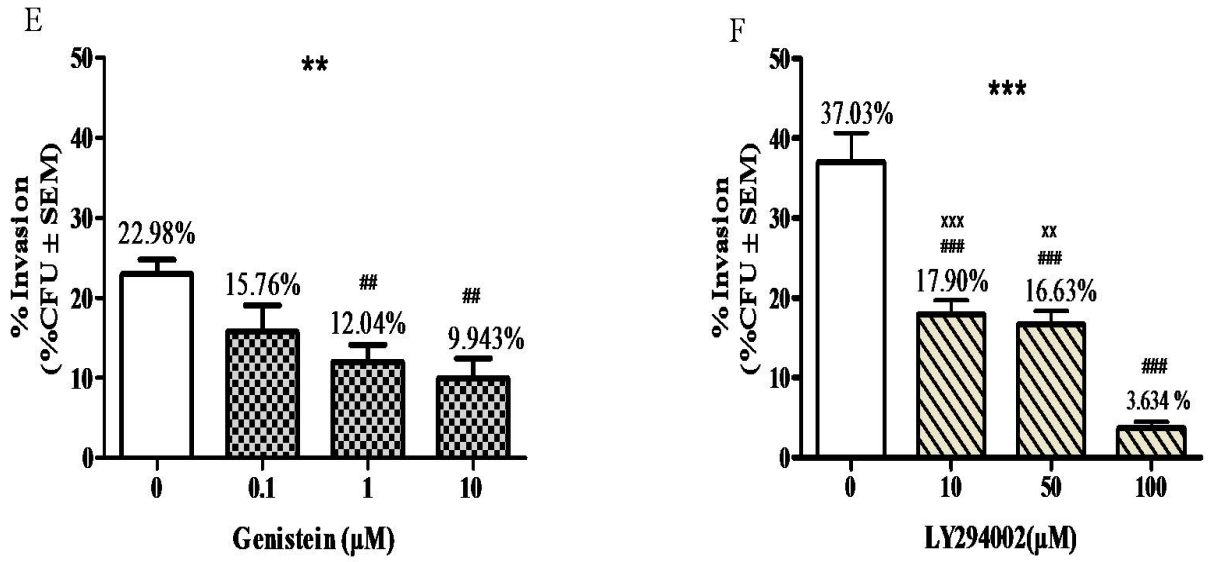


**Figure 22: Dr+ *E. coli* infection and invasion in the mIMCD3 cells treated with tyrosine kinase inhibitors.** Total bacterial infection **(A and B)** and invasion **(C and D)** were determined following 2 hrs of Dr+ *E. coli* infection in mIMCD3 cells treated with tyrosine kinase inhibitor **A and C)** genistein and **B and D)** PI3K inhibitor LY294002. Relative levels of bacterial invasion in mIMCD3 cells treated with genistein **(E)** and LY294002 **(F)** were expressed as a ratio of bacterial invasion and total bacterial infection (bacteria adhered plus bacteria internalized) in each treatment group. Data for total bacterial infection and invasion are presented as mean CFU± SEM. Relative invasion in each treatment group is expressed as % invasion and represented as % CFU internalized ± SEM for each treatment group. The error bars represent data from up to 4 experiments performed in triplicate. Data were analyzed by One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons where $P<0.05$ was considered significant. The character * indicates significant differences among the treatments, # indicates significant differences compared to the control untreated cells, and x indicates significant differences compared to 100µM LY294002 treatment.

### 7.3.2: E2 at a physiological and a pharmacological dose differentially regulates tyrosine kinase activation in mIMCD3 cells

To determine if E2-mediated differential effects on bacterial invasion involves regulation of tyrosine kinase, we analyzed the activation status of PI3K and of Akt in E2-treated mIMCD3 cells before and after Dr+ *E. coli* infection. The mIMCD3 cells were either treated with a physiological or a pharmacological dose of E2 prior to the infection and then protein levels of pPI3K and pAkt were determined in both infected as well as uninfected cells.

Treatment with a physiological dose of E2 (10 nM) led to a non-significant reduction in pPI3K and pAkt levels in both uninfected and infected mIMCD3 cells (Figures 23 and 24). Treatment with a pharmacological dose of E2 (100 nM) caused significant reductions in pPI3K and pAkt levels in uninfected cells (Figure 23A and 24A). In contrast among the infected cells, 100 nM E2 treatment induced significant increase in pPI3K and pAkt levels. Furthermore, increase in pPI3K and pAkt following infection with Dr+ *E. coli* were observed in control untreated and E2-treated mIMCD3 cells.

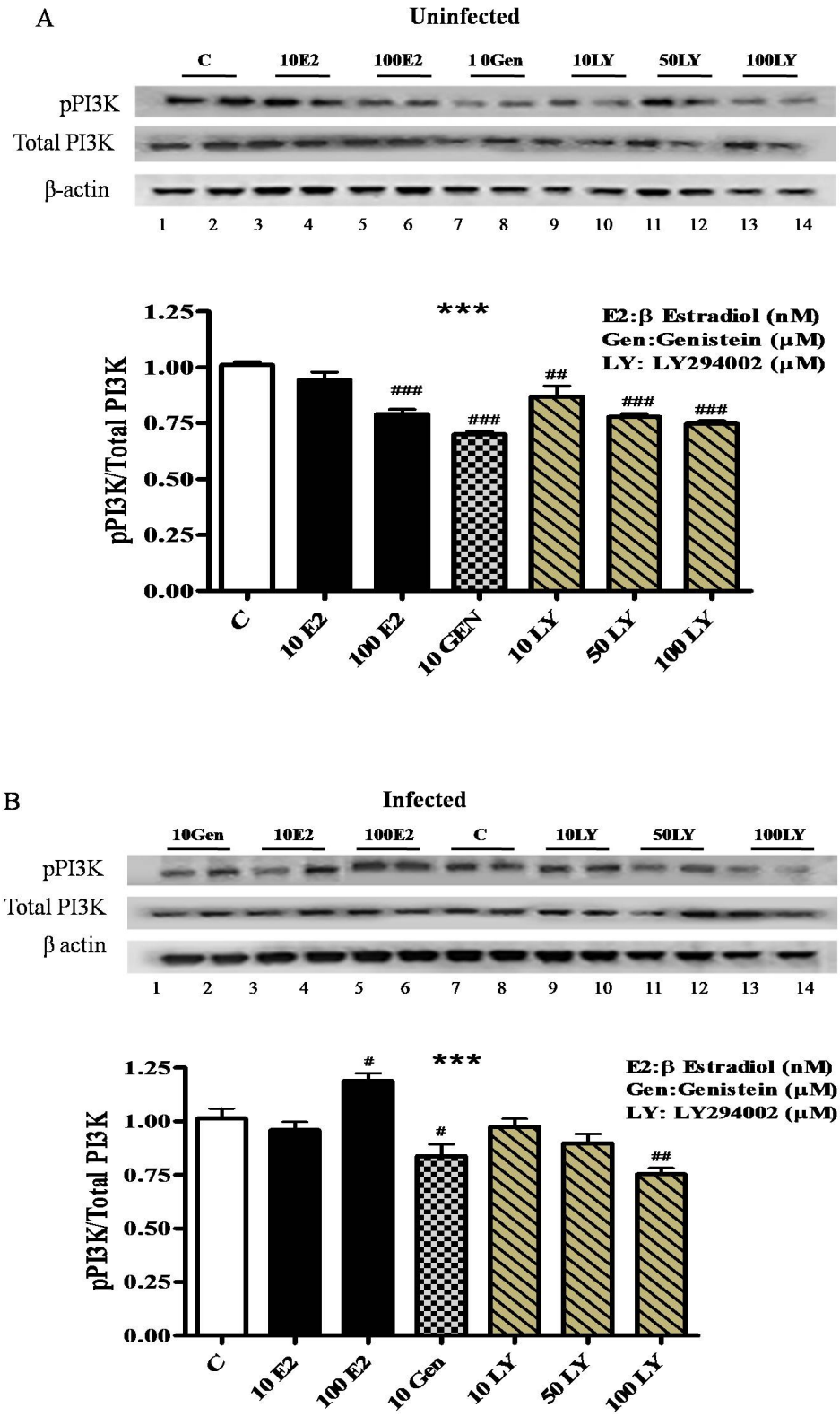
A
Uninfected
C
10E2
100E2
10Gen
10LY
50LY
100LY
pPI3K
Total PI3K
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pPI3K/Total PI3K
1.25
1.00
0.75
0.50
0.25
0.00
***
###
###
##
###
###
E2:β Estradiol (nM)
Gen:Genistein (μM)
LY: LY294002 (μM)
C
10 E2
100 E2
10 GEN
10 LY
50 LY
100 LY
B
Infected
10Gen
10E2
100E2
C
10LY
50LY
100LY
pPI3K
Total PI3K
β actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pPI3K/Total PI3K
1.25
1.00
0.75
0.50
0.25
0.00
#
***
#
##
E2:β Estradiol (nM)
Gen:Genistein (μM)
LY: LY294002 (μM)
C
10 E2
100 E2
10 Gen
10 LY
50 LY
100 LY

**Figure 23**

**Figure 23**: **Cellular levels of pPI3K in the mIMCD3 cells**. Cells were treated with E2, genistein or LY294002 and were either left uninfected **(A)** or were infected **(B)** with Dr+ *E. coli* for 2 hrs followed by cellular level detection of pPI3K using western blots. Untreated cells were considered as the control **(C)**. Protein levels of pPI3K and total PI3K in drug-treated uninfected and infected cells were determined by staining the total cell lysate with anti-pPI3-K 5(T458) Ab and anti-total PI3-K Ab, respectively. Staining for β-actin was used as a loading control. Data represent mean relative pPI3K levels ± SEM. Error bars represent data from two independent experiments performed in triplicate. The replicates from each experiment were pooled for analysis. Data were analyzed by One-Way ANOVA followed by Dunnett's post hoc test for multiple comparisons where $P<0.05$ was considered significant. The character * indicates significant differences among the treatments and # indicates significant differences compared to control untreated cells.

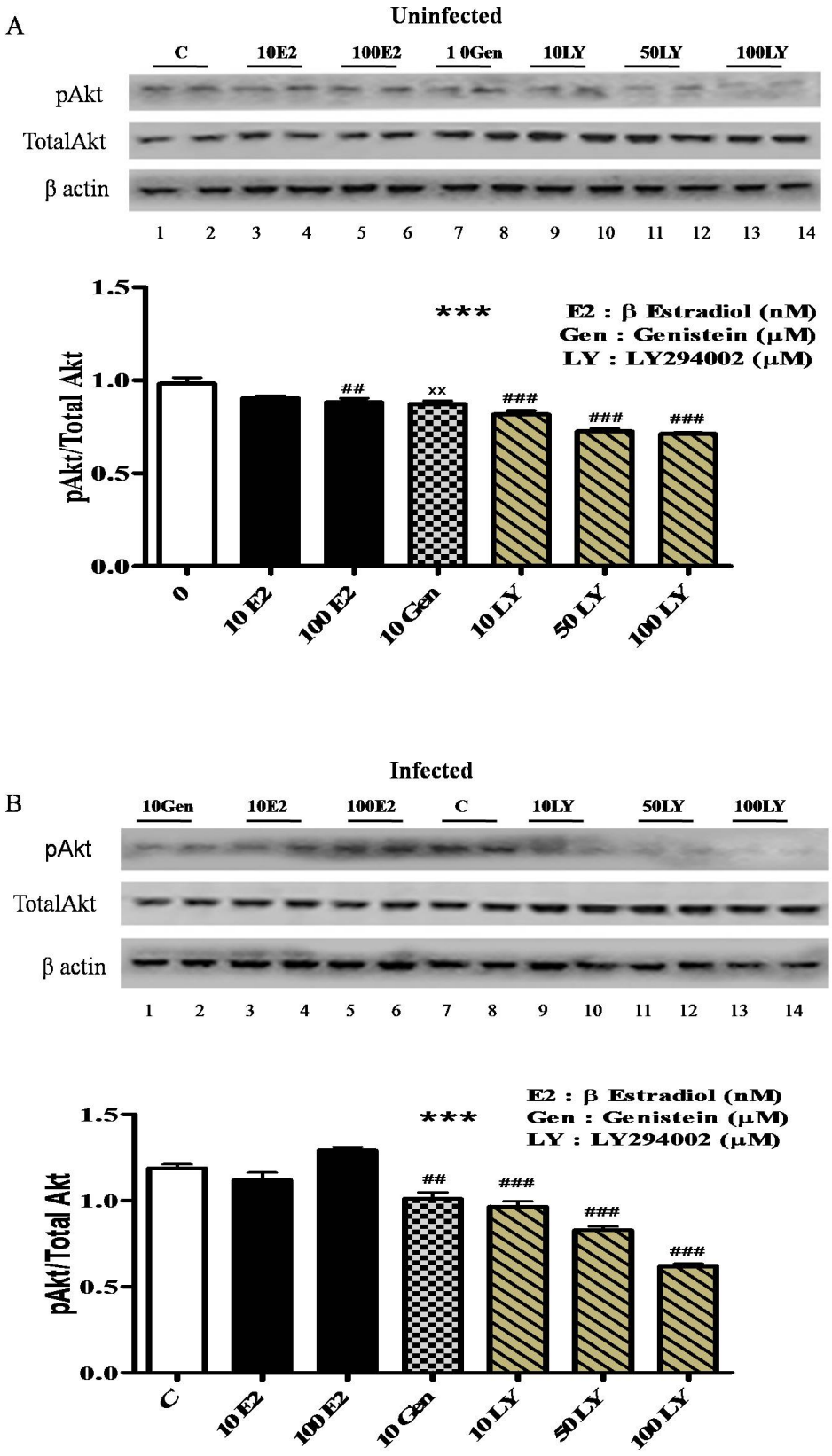
A
Uninfected
C
10E2
100E2
1 0Gen
10LY
50LY
100LY
pAkt
TotalAkt
β actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pAkt/Total Akt
1.5
1.0
0.5
0.0
***
E2 : β Estradiol (nM)
Gen : Genistein (μM)
LY : LY294002 (μM)
##
xx
###
###
###
0
10 E2
100 E2
10 Gen
10 LY
50 LY
100 LY
B
Infected
10Gen
10E2
100E2
C
10LY
50LY
100LY
pAkt
TotalAkt
β actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pAkt/Total Akt
1.5
1.0
0.5
0.0
***
E2 : β Estradiol (nM)
Gen : Genistein (μM)
LY : LY294002 (μM)
##
###
###
###
C
10 E2
100 E2
10 Gen
10 LY
50 LY
100 LY

**Figure 24**

**Figure 24**: **Cellular levels of pAkt in the mIMCD3 cells**. The mIMCD3 cells were treated with E2, or genistein, or LY294002 and were either left uninfected **(A)** or were infected **(B)** with Dr+ *E. coli* for 2 hrs followed by cellular level detection of pAkt using western blot. Untreated cells were considered as control **(C)**. Protein levels of pAkt and total Akt in drug-treated uninfected and infected cells were determined by staining the total cell lysate with anti-pAkt (S473) Ab and anti-total Akt Ab respectively. Staining for β-actin was performed as a loading control. Data represents mean relative pAkt levels ± SEM. Error bars represents data from two independent experiments performed in triplicate. The replicates from each experiment were pooled for analysis. Data were analyzed by One-Way ANOVA followed by Dunnett's post hoc test for multiple comparisons where $P<0.05$ was considered significant. The character * indicates significant differences among the treatments and # indicates significant differences compared to the control untreated cells.

## 8.4 Discussion

The results of the present study indicate that Dr+ *E. coli* infection in mIMCD3 cells induces activation of tyrosine kinase leading to phosphorylation and activation of the PI3K and Akt signaling pathways and this activation is essential for invasion of Dr+ *E. coli* into the cells. Bacterial internalization by non-phagocytic host cells involves triggering of host signal transduction mechanisms to induce rearrangements of the host cytoskeleton, thereby facilitating bacterial internalization (Martinez *et al*, 2000, Martinez & Hultgren, 2002, Munter *et al*, 2006, Yam & Theriot, 2004). Activation of tyrosine kinase involving phosphorylation and activation of the PI3K and Akt pathway appears to be a common feature leading to bacterial internalization into a variety of host cells by several invasive pathogens including UPEC (Hu *et al*, 2006, Korotkova *et al*, 2008, Martinez *et al*, 2000, Monteiro da Silva *et al*, 2007, Sandros *et al*, 1996, Uliczka *et al*, 2009). Using Huh7 hepatoma cells, we previously have demonstrated that tyrosine kinase inactivation by genistein inhibits Dr+ *E. coli* internalization (unpublished data), suggesting that a requirement for tyrosine kinase activation during Dr+ *E. coli* internalization is not limited to mIMCD3 cells. Internalization of UPEC by non-phagocytic epithelial cells requires binding of UPEC to the host cellular surface receptors. However, our results confirm that attachment to host receptors alone is insufficient to induce internalization and it is also dependent on tyrosine kinase activation. The fact that inhibition of tyrosine kinase markedly attenuated the internalization process, suggests that internalization of Dr+ *E. coli* is an active process mediated by epithelial cells.

Our results are consistent with the several other cellular signaling models of infection showing uptake of intracellular pathogenic bacteria by epithelial cells (Hu *et al*, 2006, Korotkova *et al*, 2008, Martinez *et al*, 2000, Martinez & Hultgren, 2002, Monteiro da Silva *et al*, 2007, Munter *et al*, 2006, Sandros *et al*, 1996, Uliczka *et al*, 2009, Yam & Theriot, 2004). Numerous studies have reported increase in tyrosine kinase activation following infection which is associated with bacterial uptake by the epithelial cells. Moreover, several host cellular proteins are known to participate in bacterial internalization via interacting with different host signal transduction systems. For example, activation of mitogen-activated protein kinase (MAPK) and TGF-beta signaling pathways were shown to promote invasion of *Steptococcus pneumoniae* and *Haemophilus influenzae* in nasal epithelial cells *in vitro* following infection (Beisswenger *et al*, 2007). *Campylobacter jejuni* 81-176 has been shown to interact with G protein-coupled receptor at host cell surface membrane caveolae, which triggers G-proteins and kinases to activate host proteins including PI3K and MAPK, that appear to be intimately involved in *C. jejuni* 81-176 internalization (Hu *et al*, 2006). *Staphylococcus aureus* adherence and invasion in normal human and mouse osteoblasts was found to increase the phosphorylation of the extracellular signal-regulated protein kinases (ERK 1 and 2) that correlated with MAPK activation (Ellington *et al*, 2001). Tyrosine kinases and JNK signalling were found to be crucial in *Neisseria meningitidis* invasion in human brain microvascular endothelial cells (Sokolova *et al*, 2004). Thus, we propose that the uptake of Dr+ *E. coli* by mIMCD3 cells may involve similar mechanisms that have been shown previously for internalization of Afa/Dr adhesion bearing *E. coli* and other intracellular pathogenic bacteria by non-phagocytic epithelial

cells involving host PI3K/Akt cell signal transduction activation induced engulfment of the bacterium (Donnenberg *et al*, 1994, Korotkova *et al*, 2008, Martinez *et al*, 2000, Yam & Theriot, 2004).

We further demonstrate that physiological and pharmacological E2 doses differentially regulate tyrosine kinase activation in infected and uninfected cells. Our results suggest that higher E2 dose activates tyrosine kinase signaling and this may promote increased Dr+ *E. coli* internalization in mIMCD3 cells. Physiological E2 doses attenuate tyrosine kinase activation and this may contribute to reduced bacterial internalization in mIMCD3 cells. Taken together, our results show that physiological E2 levels protect against Dr+ *E. coli* invasion by attenuating both DAF expression and tyrosine kinase activation; whereas, pharmacological E2 dose increases bacterial internalization by elevating DAF expression and tyrosine kinase activation. Activation of the PI3K/Akt pathway *in vitro* at 1 μM and higher E2 doses has been previously reported in endometrial cancer cells (Guo *et al*, 2006). Since bacterial internalization is an important feature of UTI pathogenesis, increased activation of tyrosine kinase signaling at higher E2 doses may be an important contributing factor for increased susceptibility to Dr+ *E. coli* and other UPEC induced UTIs during pregnancy. The association between increased hormonal levels during pregnancy and susceptibility to UTIs may be attributed to increased colonization receptor expression (Kaul *et al*, 1999) and tyrosine kinase-activated bacterial internalization (Wroblewska-Seniuk *et al*, 2005) induced by elevated estrogen levels. Blockade or attenuation of PI3K/Akt signaling cascade may be

considered as potential therapeutic approaches for effective control of bacterial internalization during UTI pathogenesis in hosts.

To our knowledge, no other studies to date have shown estrogen-mediated regulation of tyrosine kinase activation during bacterial internalization. Our study indicates ERα to be the functional ER subtype regulating DAF expression and Dr+ *E. coli* internalization in mIMCD3 cells. We have not investigated the ER subtype involved in regulating E2-mediated tyrosine kinase activation during Dr+ *E. coli* internalization in mIMCD3 cells. Since ERα is the predominant subtype expressed in mIMCD3 cells, it is expected that tyrosine kinase activation by E2 in these cells may be regulated by ERα, however, studies will be conducted in future to discern the ER subtype involved in this estrogen-induced response. Nonetheless, ERα-mediated estrogenic regulation of PI3K/Akt signaling activation has been previously indicated (Castoria *et al*, 2001).

Non-genomic signaling by estrogen involves rapid activation of cellular signaling kinases via activated ERs resulting in transcriptional regulation of target genes (Cato *et al*, 2002, Edwards & Boonyaratanakornkit, 2003, Hammes & Levin, 2007, Mannella & Brinton, 2006). For instance, in MCF7 cells E2-mediated regulation of PI3K signaling has been shown to regulate gene transcription via MAPK activation (Migliaccio *et al*, 1996). Moreover, we have previously shown that estrogen-mediated protection against Dr+ *E. coli* internalization in Huh-7 hepatoma cells and primary human hepatocytes involves regulation of MAPK activation (Kaul *et al*, 2007) and DAF expression (unpublished data). However, we have not yet investigated the effects of PI3K/Akt

signaling or PI3K/Akt and MAPK interaction on DAF expression. Our previous observations and results from this study indicate that E2-mediated transcriptional regulation of DAF in mIMCD3 cells may possibly include regulation by classical ER dependent genomic pathways as well as signaling activation of MAPK or other related pathways via PI3K/Akt activation, and this will be further investigated.

We, conclude that binding of Dr+ *E. coli* to DAF on mIMCD3 surfaces entails activation of PI3K and Akt via tyrosine kinase signaling pathway and the uptake of Dr+ *E. coli* in mIMCD3 cells during infection is dependent on tyrosine kinase activation. A dichotomy in the effects of E2 on tyrosine kinase activation at physiological as well as pharmacological doses is observed. This may influence the differential modulation of the process of bacterial internalization at varying E2 doses. The detailed mechanism for estrogen-mediated regulation of cellular signaling needs to be explored to delineate the signaling pathways involved in bacterial colonization and internalization in host epithelium. The information obtained can be further exploited to find therapeutic applications against UTIs.

# CHAPTER VIII

# ESTROGEN IS REQUIRED FOR PROPER INFLAMMATORY RESPONSE AGAINST INFECTION IN THE KIDNEY

## 8.1 Introduction

Inflammation is a multicomponent response to infection and tissue injury that functions to defend the host against infection and provides tissue remodeling and repair. The most distal renal tubule segments, the medullary collecting ducts are the first to come in contact with the ascending UPEC. Thus, IMCD are the primary site for bacterial adherence and early inflammatory responses against UPEC. UPEC activate TLRs expressed on the renal tubular epithelial cells and induce adequate immune responses in the host. TLR activation up-regulate secretion of pro-inflammatory cytokines (TNFα. IL6, IL-1β) and chemokines ( MIP-2, RANTES, MCP-1), contributing to UPEC clearance (Chassin *et al*, 2006, Patole *et al*, 2005, Samuelsson *et al*, 2004, Schilling *et al*, 2001b, Tsuboi *et al*, 2002, Wolfs *et al*, 2002, Wullt *et al*, 2001). In the renal tubules, TLR4 are considered to be the key inflammation inducer against the gram negative UPEC. Additionally, activation of other TLRs including TLR2, TLR5, TLR 6 and TLR11 has also been shown to induce inflammatory response against UPEC (Chassin *et al*, 2006, Vandewalle, 2008). Furthermore, expression of TLR2 and TLR4 has been

observed to be up-regulated in the distal nephrons of the inflammed kidneys following injury and during UTIs (El-Achkar & Dagher, 2006, Gluba *et al*, 2010, Wolfs *et al*, 2002).

Recent studies have shown that pro-inflammatory responses are crucial for providing the first line of defense against invading pathogen in the urinary tract. Nonetheless, overwhelming activation of TLR signaling and excessive inflammatory responses are deleterious. Heightened inflammation can induce cellular damage in the kidney, causing the development of chronic pyelonephritis and tubulointerstitial nephritis. This may further lead to impaired kidney function and ultimately kidney fibrosis and kidney failure. Activation of TLRs under normal physiological condition appears to be tightly regulated and at present various mechanisms employed in regulation of TLR triggered inflammatory immune responses are under study (O'Neill, 2008, Wang *et al*, 2009).

The kidneys of estrogen-deficient OVX mice had more colonization with Dr+ *E. coli* compared to the kidneys of OVX mice that were pretreated with E2 at a physiological dose. The kidneys of ERα-/- mice also showed increased Dr+ *E. coli* colonization compared to the kidneys of the ERα+/+ mice. These results indicate E2/ERα mediated protection against UTIs. Furthermore, OVX mice pretreated with E2 at a physiological dose have reduced kidney inflammation compared to kidney inflammation observed in the vehicle treated OVX mice post 21 days of Dr+ *E. coli* infection *(*preliminary data, Figure 2), suggesting induction of chronic inflammation in estrogen deficient mice post-infection. It appears that E2/ERα play an important role in the immunoregulation of

inflammatory responses against UTIs in the host. The present study was conducted to determine the effects of E2/ERα on the immune responses post UTI-induction *in vivo* and *in vitro*. We measured the mRNA levels of TLR 2 and TLR 4 and pro-inflammatory cytokines (TNFα and IL6) and chemokine (MIP2) in the kidneys of OVX C3H/HeJ and the ERα-/- B6.129 mice post-UTI induction. The mRNA levels of these genes were also determined in mIMCD3 cells, following infection with Dr+ *E. coli*

Estrogen-deficient OVX C3H/HeJ and ERα-/- mice show depressed immune responses during the onset of infection and show heightened inflammatory responses at later time points post-infection. In contrast, the respective control group of estrogen-sufficient Non-OVX C3H/HeJ mice and ERα+/+ B6.129 mice show rapid inflammatory responses at 2 days post-infection, immediately followed by a decline in inflammatory responses at a later time point post-infection. Dr+ *E. coli* infection of mIMCD3 cells for 2 hrs caused increased inflammatory responses in the mIMCD3 cells. Estrogen treatment of cells resulted in reduction of inflammatory responses; however the reduction was not statistically significant.

## 8.2 Statement of hypothesis

Estrogen, via ERα, regulates inflammatory response in the mouse kidney against Dr+ *E. coli* infection promoting rapid bacterial clearance and reducing inflammation mediated tissue damage.

## 8.3 Results

### A) *In vivo* results

### 8.3.1: Estrogen deficiency leads to delayed inflammatory responses in the kidneys of OVX C3H/HeJ mice post-infection

To understand the role of estrogen in regulating inflammation following UTI induction, we investigated the effect of estrogen-deficiency on the host's inflammatory responses against Dr+ *E. coli* infection in OVX C3H/HeJ mice. The mRNA levels of inflammation inducer, TLR2, pro inflammatory cytokines, TNFα and IL6, and chemokine, MIP2 were measured in the kidneys of uninfected and Dr+ *E. coli* infected OVX and Non-OVX mice. C3H /HeJ mice are defective in TLR4 signaling therefore we analyzed the expression of only TLR2.

The kidneys of uninfected Non-OVX mice expressed higher levels of TLR2 mRNA compared to the the kidneys of OVX mice, but the difference was not statistically significant. In the infected mice, at 2 and 6 days post-UTI induction TLR2 mRNA levels in the OVX and the Non-OVX mice were comparable to those in the uninfected mice. However, at 21 days post- infection TLR2 mRNA levels were found to be significantly increased in both, the OVX and Non-OVX C3H/HeJ mice compared to those in the uninfected controls (Figure 25A). Among the infected group, TLR2 expression remained decreased in the OVX mice compared to that in the Non-OVX mice at both 2 and 6 days

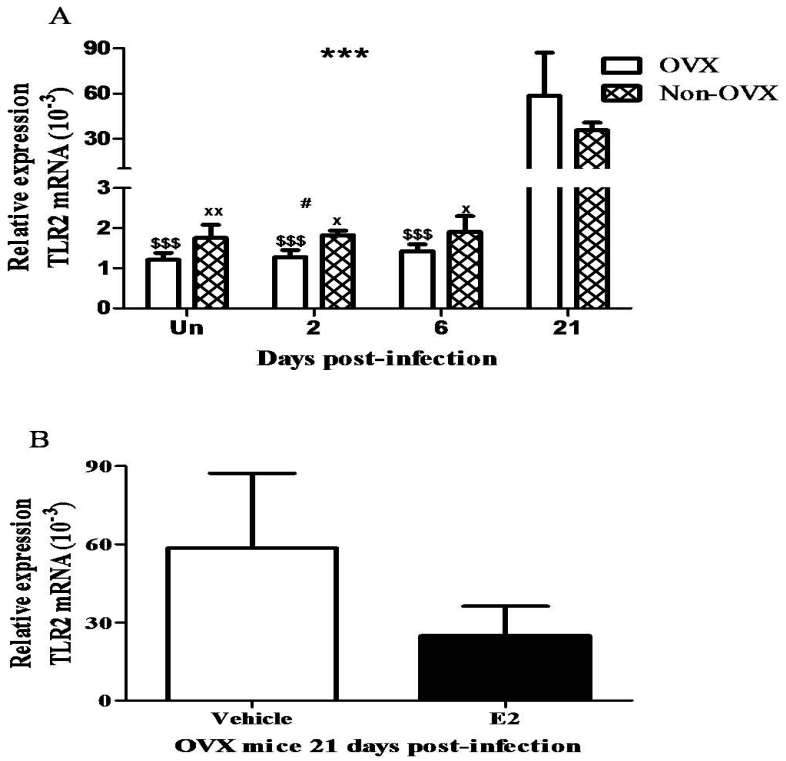


**Figure 25: TLR2 and pro-inflammatory cytokines mRNA levels in the kidneys of OVX and Non-OVX C3H/HeJ mice.** Target gene mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Target mRNA levels were normalized to PPIA mRNA levels in each sample and relative expression was calculated. Expression values are mean relative expression ± SEM and the error bars represent data from at least 5 animals in each study group.

**A) TLR2 mRNA levels in the kidneys of uninfected (Un) and Dr+ *E. coli* infected OVX and Non-OVX mice at 2, 6 and 21 days post-UTI induction.** Data were analyzed by Two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons where $P < 0.05$ was considered significant. The character * represents significant difference among the study groups, # represents significant difference between OVX and Non-OVX mice, $ represents significant difference from 21 day infected OVX mice and x represents significant difference from 21 day infected Non-OVX mice.

**B) TLR2 mRNA levels in the kidneys of Dr+ *E. coli* infected E2 and vehicle treated OVX mice 21 days post-UTI induction.** Data were analyzed by Student's T test where $P < 0.05$ was considered significant.

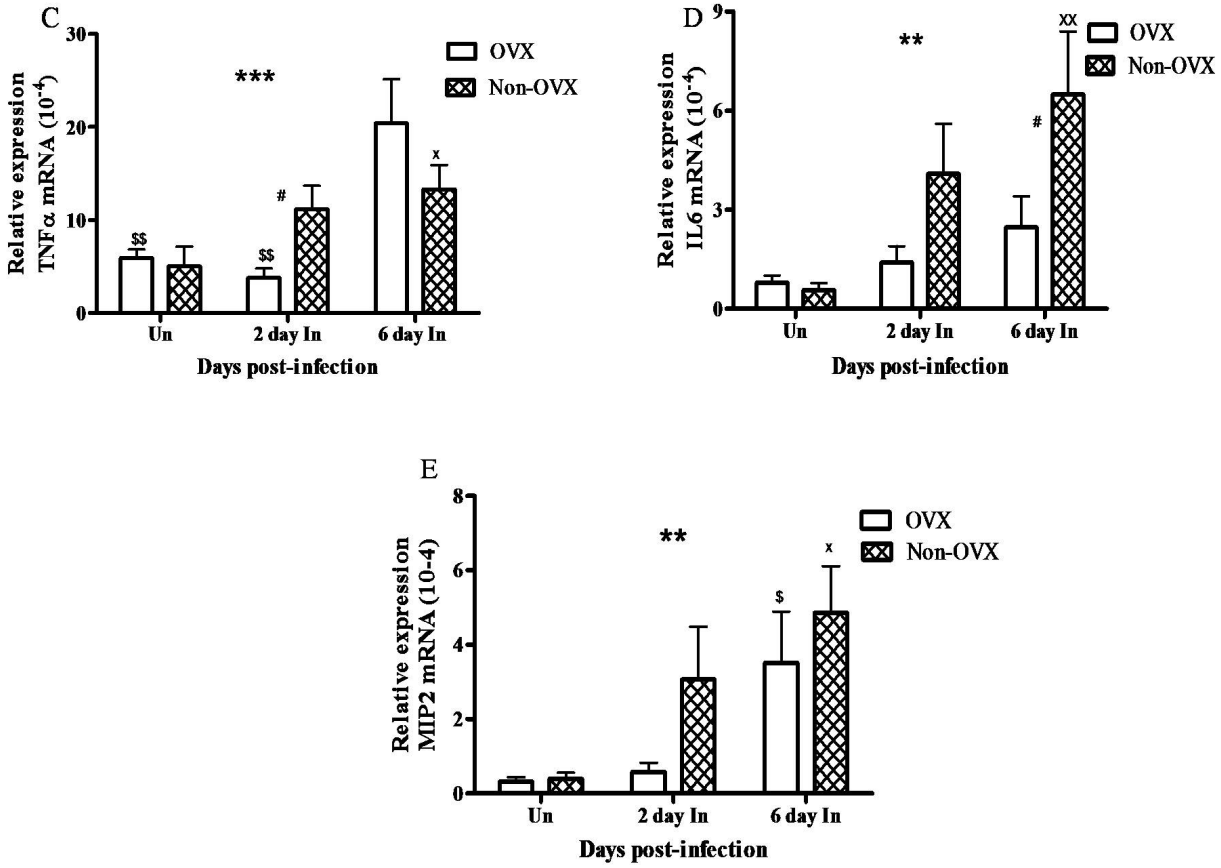


**Figure 25: C-E) Levels of pro inflammatory cytokines TNFα (C), IL6 (D), and MIP2 (E) mRNA in the kidneys of uninfected (Un) and Dr+ *E. coli* infected OVX and Non-OVX mice at 2, and 6 days post- UTI induction.** Data were analyzed by Two-Way ANOVA followed by Bonferroni post hoc test for multiple comparisons where $P< 0.05$ was considered significant. The character * represents significant difference among the study groups, # represents significant difference between OVX and Non-OVX mice, $ represents significant difference from 21 day infected OVX mice and x represents significant difference from 21 day infected Non-OVX mice.

post-infection. However, at 21 days post- infection TLR2 mRNA expression was observed to be increased by 2-fold in the OVX mice compared to that in the Non-OVX mice. The E2-treated OVX mice also showed 2-fold reduced expression of TLR2 compared to that in the vehicle-treated mice (Figure 25B), however the differences were not found to be staitistically significant.

Infection increased cytokine mRNA in the kidneys of both the OVX and the Non-OVX mice. Among the infected group of mice, time specific serial increase of cytokine mRNA was found in OVX, as well as Non-OVX mice, at 3 versus 7 days post-UTI induction. However, in the OVX mice significant increase in TNFα, IL6, or MIP2 mRNA was observed only at 6 days post-infection. In contrast, in the Non-OVX mice, expression of these cytokine mRNA were found to be markedly elevated at 2 days post infection and their levels increased significantly at 6 days post infection (Figure 25C). Notably, at both 3 and 7 days post-infection, the OVX mice expressed reduced cytokine mRNA levels compared to the Non- OVX mice. However at 7 days post–infection the TNFα mRNA levels in the kidneys of OVX mice were found to be increased compared to those in the kidneys of Non-OVX mice (Figure 25C).

### 8.3.2: ERα-/- mice mount delayed inflammatory responses than do the ERα+/+ mice post-infection

To determine the specific role of ERα in regulating the kidney immune responses against Dr+ *E. coli* in the infected kidneys, we measure the mRNA levels of TLR4,

TLR2, TNFα, IL6, and MIP2 in the kidneys of ERα-/- and ERα+/+ mice at 3 and 7 days post-UTI induction.

The expression of TLR2, TLR4 and pro-inflammatory cytokines in the ERα-/- and ERα+/+ mice are represented in Figure 26. Time specific serial increases in TLR2 and TLR4 mRNA levels at 3 verses 7 days post-infection were found in ERα-/- mice (Figure 26 A-B). In contras, ERα+/+ mice showed a serial decrease in the TLR2 mRNA levels from 3 to 7 days post–infection (Figure 26A). TLR4 expression in the ERα+/+ mice remained similar at 3 and 7 days post-infection (Figure 26B).

At 3 vs 7 days post infection, we observed time specific serial increases in TNFα and IL6 mRNA in the ERα-/- mice. In contrast, there was a serial decrease in the mRNA levels of these cytokines in the ERα+/+ mice from 3 to 7 days post-infection (Figures 26C and 26E). Importantly, the serum TNFα levels in the ERα-/- were also found to be significantly higher compared to that in the ERα+/+ mice at7 days post-infection (Figure 26 D). In comparison to the the ERα+/+ mice, the ERα-/- mice expressed higher levels of MIP2 mRNA at 3, as well as 7 days, post-UTI induction (Figure 26F).

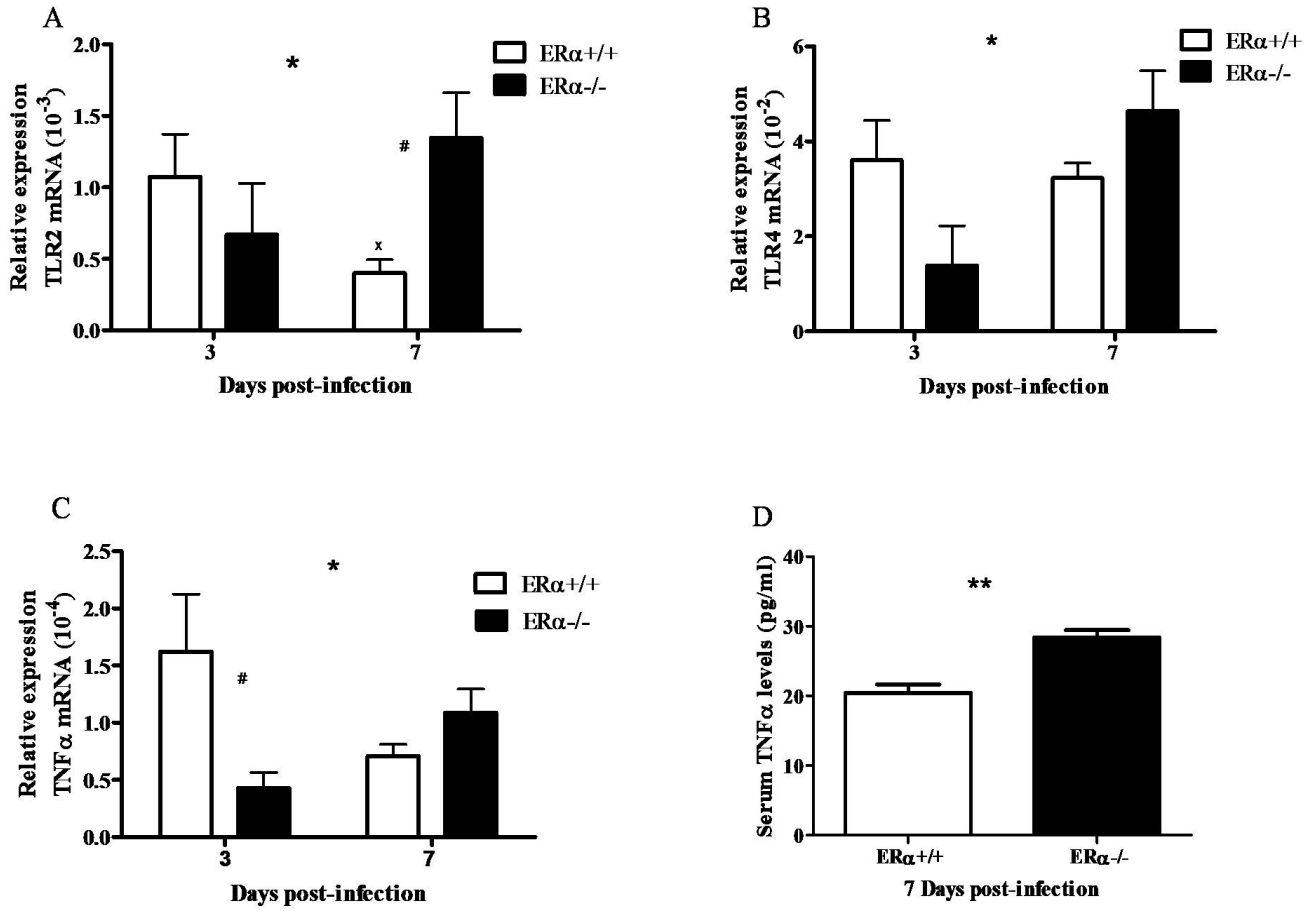


**Figure 26**

**Figure 26: TLR2, TLR4 and pro-inflammatory cytokines mRNA levels in the kidneys of ERα-/- and ERα+/+ mice.** Target gene mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Target mRNA levels were normalized to PPIA mRNA levels in each sample and relative expression was calculated. Expression values are mean relative expression ± SEM and the error bars represent data from at least 4- 5 animals in each study group.

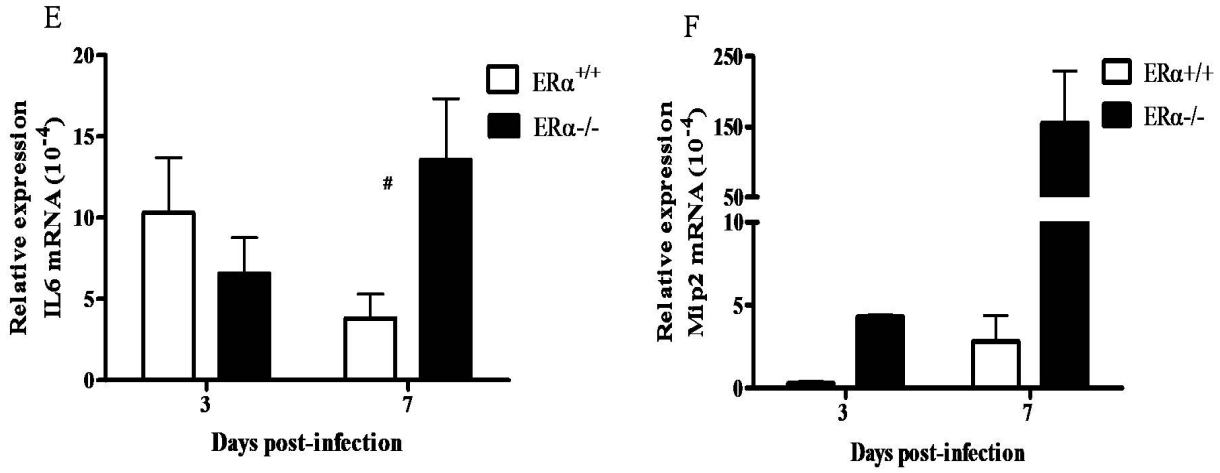


**Figure 26: TLR2, TLR4 and pro-inflammatory cytokines mRNA levels in the kidneys of ERα-/- and ERα+/+ mice**

**A-B) TLR2 and TLR4mRNA levels in the kidneys of ERα-/- and ERα+/+ mice at 3 and 7 days post-UTI induction.** Data were analyzed by Two-Way ANOVA followed by Bonferroni post hoc test for multiple comparisons where P< 0.05 was considered significant. The character * represents significant difference among the study groups, # represents significant difference between ERα-/- and ERα+/+ mice, and x represents significant difference between 3 and 7 day infected ERα+/+ mice.

**C-D) TNFα expression in kidneys of Dr+ *E. coli* infected ERα-/- and ERα+/+ mice post-UTI induction at mRNA (C) and protein (D) levels.** Data were analyzed by Two-Way ANOVA followed by Bonferroni post hoc test for multiple comparisons for Figure C and Student's t test for Figure D where P< 0.05 was considered significant. The character * represents significant difference among the study groups, # represents significant difference between ERα-/- and ERα+/+ mice.

**E-F) Levels of pro inflammatory cytokines, IL6 (D) and MIP2 (E) mRNA in the kidney of ERα-/- and ERα+/+ mice at 3 and 7 days post-UTI induction.** Data analyzed by Two-Way ANOVA followed by Bonferroni post hoc test for multiple comparisons where P< 0.05 was considered significant.

**B) *In vitro* results**

### 8.3.3. Effects of E2 on inflammatory responses *in vitro* in mIMCD3 cells post-infection.

We determined the effect of estrogen at different E2 doses on the mRNA levels of TLR2 and 4, pro-inflammatory cytokines (TNFα, IL6), and chemokines (MIP2) in the uninfected and Dr+ *E.* coli infected mIMCD3 cells following 2 hrs of infection.

Infection significantly increased the mRNA levels of TLR2, TLR4, TNFα, IL6, and MIP2 in both E2-treated, as well as untreated cells (Figure 27). At 2 hrs post-infection no significant difference was observed in mRNA levels of these inflammatory markers in E2 treated or untreated mIMCD3 cells.

## 8.4 Discussion

To our knowledge we demonstrate for the first time, the modulatory effects of estrogen on pro-inflammatory responses in the kidneys of mice challenged with Dr+ *E. coli*. We also show that the estrogen-mediated regulation of innate immune response in the kidney is mediated via action of ERα activation pathway. Our time point data following Dr+ *E. coli* infection in the OVX mice showed delay in induction of innate immune responses in the kidneys. In contrast, the Non-OVX mice exhibited induction of

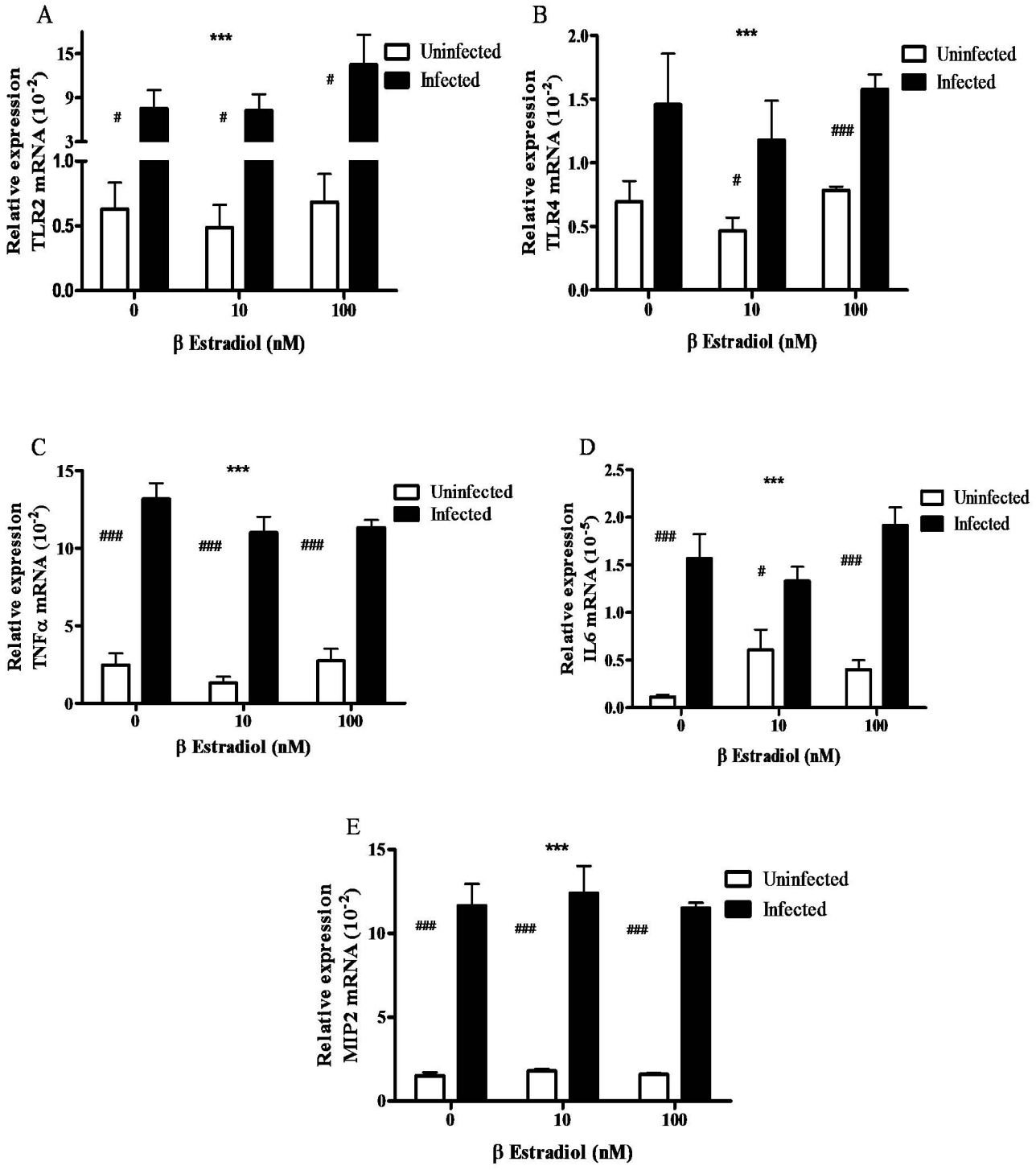
A
Relative expression TLR2 mRNA ($10^{-2}$)
Uninfected
Infected
β Estradiol (nM)
B
Relative expression TLR4 mRNA ($10^{-2}$)
C
Relative expression TNFα mRNA ($10^{-2}$)
D
Relative expression IL6 mRNA ($10^{-5}$)
E
Relative expression MIP2 mRNA ($10^{-2}$)

**Figure 27**

**Figure 27 TLR2, TLR4, and pro-inflammatory cytokines mRNA levels in E2-treated mIMCD3 cells following infections with Dr+ *E. coli* for 2 hrs.** Target gene mRNA levels were determined by quantitative SYBR green real-time RT-PCR using gene specific primers. Target mRNA levels were normalized to PPIA mRNA levels in each sample and relative expression was calculated. Expression values are mean relative expression ± SEM and error bars represent data from at least 4 experiments performed in triplicate. Data analyzed by Two-Way ANOVA followed by Bonferroni post hoc test for multiple comparisons where $P<0.05$ was considered significant. The character * represents significant difference among the study groups, and # represents significant difference between uninfected and infected cells.

robust inflammatory responses following the onset of infection. We have earlier demonstrated that estrogen at a physiological dose provides protection against Dr+ *E. coli* colonization, whereas estrogen deficiency increases Dr+ *E. coli* colonization in the mice kidneys (results discussed in chapter IV). Based on these results, it appears that differential inflammatory responses are generated in the kidneys in the presence or absence of estrogen as observed in these mice and may contribute to differential bacterial colonization. Furthermore, following UTI induction in the ERα-/- mice, we found a similar depressed and delayed immune responses in the kidneys that may be responsible for increased bacterial colonization in these mice (discussed in chapter IV). On the contrary, similar to Non-OVX mice ERα+/+ mice were able to generate rapid immune responses resulting in clearance of infection and regulation of the inflammatory responses earlier compared to the ERα-/- mice. Thus, apart from proinflammatory cytokines, function of ERα is important for regulating infection and inflammation against Dr+ *E. coli* in the kidney.

Following infection, bacterial colonization in the urinary tract is controlled by TLR activation and pro-inflammatory cytokine up-regulation (Svanborg *et al*, 2001a, Svanborg *et al*, 2001b). The bladder and kidney epithelial cells are reported to be the major sources of IL6 and IL 8 following infection with UPEC (Hedges *et al*, 1994, Hedges *et al*, 1996). IL6 serves a variety of immunoregulatory functions including amplification of inflammatory signals involved in neutrophil recruitment and stimulation of IgA secretion by B cells (Hedges *et al*, 1991, Hedges *et al*, 1992). In the human urinary tract IL8 attracts the neutrophils and a similar role is played by MIP2 in the

murine urinary tract (Hang *et al*, 1999). TNFα initiates the inflammatory signaling cascade by TLR2 and TLR4 up-regulation, as well as induction, of IL6 and IL8/MIP2 secretion from the uroepithelial cells (Billips *et al*, 2007, Wolfs *et al*, 2002). In our study, the estrogen deficient OVX mice and the ERα-/- mice had reduced stimulation of these pro-inflammatory cytokines during the early stages of UPEC infection that correlated with increased bacterial colonization in their kidneys. Also, the ERα-/- mice exhibited lack of regulation of proinflammatory responses and continued to have higher levels of cytokines at the later time point compared to the that in the ERα+/+mice. TNFα but not IL6 or MIP2 were found to be expressed more in the OVX mice kidneys than in the Non-OVX mice kidneys, at day 6 post-infection. This indicates a differential regulation of TNFα by estrogen.

We found a delay in increase of TLR2 expression in the OVX, as well as the Non-OVX C3H/HeJ mice post-infection, and an increase in TLR2 expression was found only at 21 days post-infection. The delay in TLR2 activation at early time points may be responsible for the increased bacterial colonization and elevated inflammation in the OVX mice at a later time point compared to those in the Non-OVX and E2-treated OVX mice. TLR2 expression is suggested to be dependent on inflammatory cytokines priming (Wolfs *et al*, 2002). C3H/HeJ mice are defective in TLR4 signaling resulting in LPS unresponsiveness and reduced inflammatory signaling (Haraoka *et al*, 1999, Shahin *et al*, 1987), which may explain the delay in up-regulation of TLR2 in these mice. Despite the delayed immune resposes, the Non-OVX and E2-treated OVX C3H/HeJ mice were found to express increased levels of TLR2 compared to the OVX C3H/HeJ mice at earlier time

points following infection, suggesting estrogen mediated regulation of TLR2. However, at 21 days post-infection, Non-OVX mice and E2-treated OVX mice expressed reduced TLR2 mRNA levels compared to that in the OVX mice. Furthermore, in the E2-treated group of mice we found significantly reduced levels of serum TNFα, IL6, MIP 1α, and other pro-inflammatory cytokines at 21 days post-UTI induction (unpublished data). Supporting this data on E2 effects, at 21 days post-UTI induction the kidney tissues of E2-treated OVX mice were also found to have significantly reduced inflammation and lymphocyte infiltration compared to those observed in the kidneys of vehicle- treated OVX mice (preliminary data, Figure 2). Thus, physiological levels of estrogen in mice seems to be necessary for initial rapid stimulation of TLR activation and pro-inflammatory cytokine secretion resulting in bacterial clearance and further to limit the inflammatory response at the later stages of infection to prevent tissue damage associated with sustained inflammation. It is possible that estrogen may also stimulate anti-inflammatory effects as we have found down-regulation of TLR and pro-inflammatory cytokine secretion at later stages of infection in estrogen sufficient mice. However in this study we have not assessed the expression of anti-inflammatory cytokines.

Modulatory effects of estrogen have been studied in several animal models of chronic inflammatory diseases and in kidney damage (Blush *et al*, 2004, Elliot *et al*, 2007, Satake *et al*, 2008, Silbiger & Neugarten, 2008, Straub, 2007). Our results are in accordance with the study, where physiological dose of E2 via ERα was found to be necessary for rapid induction of TLR2, TNFα, and IL-12 following bacterial or viral infections in female mouse brain (Soucy *et al*, 2005). In addition, estrogen at physiological levels was

found to enhance the antibacterial activity in secretions of polarized human uterine epithelial cells (ECC-1) in culture (Fahey *et al*, 2008). Moreover, the proestrous state of estrous cycle in females is characterized by more vigorous immune responses compared to the diestrous stage and to the males (Angele *et al*, 2006). Also, proestrous female mice have maintained splenocyte function and tolerate polymicrobial sepsis much better than do the male mice that exhibit depressed immune responses under those conditions, indicating a possible role of estrogen in enhanced immune responses in females (Zellweger *et al*, 1997). Depressed release of pro-inflammatory cytokines is also observed in aged female mice following trauma haemorrhage and this observation is in accordance with the clinical finding demonstrating higher mortality rates in septic postmenopausal women (Angele *et al*, 2003, McLauchlan *et al*, 1995). Furthermore, immune cells also express ERs (Straub, 2007), suggesting that estrogen may influence the immune cells within the tissue of the affected organ to modulate inflammatory responses. For example physiological E2 levels were found to directly modulate dendritic cell differentiation (Carreras *et al*, 2008, Carreras *et al*, 2010). Estrogen deficiency has been associated with increased TNFα production by T cells leading to increased osteoclast formation and bone resorption, furthermore estrogen is suggested to prevent bone loss by regulating T cell function and immune cell bone interactions (Weitzmann & Pacifici, 2006). Additionally, estrogen deficiency has been shown to up-regulate TLR2 and TLR 4 expression in osteoclast cells and epidermal keratinocytes; furthermore, presence of estrogen has also been shown to up-regulate TLR signaling (Kim *et al*, 2009, Koh *et al*, 2009, Moeinpour *et al*, 2007). Our findings support the results from these studies showing importance of estrogen in immune modulation and in maintenance of host

immune homeostasis following infection. Thus far, there are limited studies reporting estrogen mediated regulation of TLR expression. Our study provides the first *in vivo* evidence for estrogen/ERα-mediated regulation of TLR2 and TLR4 in the kidney during Dr+ *E. coli* induced UTI.

In mIMCD3 cells, following 2 hrs of infection we found increased expression of TLRs and pro-inflammatory cytokines. For uninfected, as well as infected cells treated with physiological dose of E2 (10 nM), we found a slight decrease in expression of inflammatory markers except for TLR2 and MIP2, however the decrease was not statistically significant. Lack of time course experiments for E2 treatment in this study are responsible for inconclusive data. Infection and cytokine kinetics experiments will be performed in the future to better determine the effects of estrogen on expression of TLRs and pro-inflammatory cytokines using our *in vitro* mIMCD3 cells culture model.

Inflammation is also associated with increased expression of DAF and type IV collagen that are the receptors for Dr+ *E. coli* colonization. The basal expression of DAF and type IV collagen expression in the uninfected mice are higher in OVX than in Non-OVX mice, suggesting E2 mediated regulation of these receptors. Our studies, as well as other *in vivo* and *in vitro* studies have shown inflammation induced elevated expression of DAF and collagen expression following infection (Betis *et al*, 2003b, Fang *et al*, 2004, Mankhey *et al*, 2007, Meldrum *et al*, 2007, Nowicki *et al*, 2009, Potier *et al*, 2002). In the present study following 2 days of infection, the Non-OVX and the ERα+/+ mice expressed higher levels of cytokines that corresponded with relatively higher DAF and

type IV collagen mRNA expression (discussed in chapter VI). Supporting our argument, we found higher Dr+ *E. coli* colonization initially in the Non-OVX mice and in the ERα+/+ at this time point. However at a later time point the expression of pro-inflammatory cytokines, DAF, and type IV collagen decreased in the estrogen sufficient mice. These mice also showed reduced bacterial colonization compared to the OVX mice at later stages post-infection.

Pathogen recognition by mucosa and subsequent release of pro inflammatory cytokines are critical features of the innate host defense system against invading pathogens. Our results support our hypothesis that physiological estrogen levels regulate bacterial colonization and the resulting innate inflammatory responses thus promoting rapid bacterial clearance and limiting inflammation induced tissue damage in Dr+ *E. coli* infected mouse kidney. Furthermore, ERα action is involved in this protection. Additional studies are needed to be conducted to understand the effects of estrogen at different cellular levels in modulating inflammatory responses and to study its role in shifting the immune responses from innate to adaptive during pathogen elimination.

# CHAPTER IX

# SUMMARY AND SIGNIFICANCE

## 9.1 Summary and Conclusion

The pathogenesis of UTIs is complex and is influenced by various virulence factors of the UPEC as well as by several host factors. Possible role of ovarian hormones, particularly estrogen has been implicated in the etiology of UTIs in females. However, the precise mechanism associated with estrogen related etiology of UTIs is unclear. Limited *in vivo* and *in vitro* studies have been conducted in this area. By utilizing our *in vivo* experimental UTI mouse model and *in vitro* mIMCD3 cell culture model, we investigated the possible effects of estrogen deficiency and physiological dose of estrogen on susceptibility to Dr+ *E. coli* uropathogenesis. We also determined the role of ER subtype in mediating the possible effects of estrogen during Dr+ *E.coli* infection using our *in vitro* as well as *in vivo* experimental UTI models. To our knowledge, this study provides the first *in vitro* and *in vivo* evidences for estrogen and ERα mediated modulation of Dr+ *E. coli* uropathogenesis. The results and conclusions from our *in vivo* and *in vitro* studies are summarized below.

### *A) In vivo* studies

#### 9.1.1: Estrogen-deficiency in OVX C3H/HeJ mice increases Dr+ *E. coli* colonization in the urinary tracts, and estrogen supplementation at a physiological dose in these mice provides protection against Dr+ *E. coli* colonization in the urinary tracts.

The estrogen-deficient OVX mice showed increased susceptibility to Dr+ *E. coli* colonization in the bladder and kidneys compared to the colonization in the bladders and kidneys of the estrogen-sufficient Non-OVX mice. Additionally, treatment of the OVX mice with a physiological dose of E2, prior to UTI induction, led to a reduced bacterial colonization in their bladder and kidneys. These observations suggest that estrogen at a physiological dose serves as an important host factor in providing protection against Dr+ *E. coli* colonization in the urinary tract.

#### 9.1.2: Protective effects of a physiological dose of E2 in mouse kidney include regulation of DAF and type IV expression, and modulation of infection induced inflammation.

The estrogen-deficient OVX mice expressed increased levels of Dr+ *E. coli* colonization receptors, DAF and type IV collagen, in the kidneys compared to the expression of these receptors in the Non-OVX mice, suggesting increased susceptibility of OVX mice to Dr+ *E. coli* induced UTI. Following infection, the OVX mice treated with a physiological dose of E2 and the estrogen sufficient Non-OVX mice expressed reduced levels of DAF and type IV collagen in the kidneys compared to the expression of

these receptors in the vehicle-treated OVX mice. These observations led us to conclude that host endogenous estrogen at host physiological dose down-regulate DAF and type IV collagen expression in the kidney, thus allowing reducing Dr+ *E. coli* colonization.

The inflammatory responses in the estrogen-sufficient Non-OVX C3H/HeJ mice at the onset of infection were found to be elevated. In contrast, inflammatory responses in the estrogen-deficient OVX mice were found to be depressed which correlated with increased bacterial colonization in the kidneys of these mice. The inflammatory responses in the estrogen-sufficient Non-OVX mice and the E2-treated OVX mice declined at latter time point; however the inflammatory responses in the estrogen-deficient OVX mice increased profoundly. These observations suggest that a physiological level of estrogen is an important host factor for resolution of infection as it promotes rapid induction of optimum immune response. In contrast, host estrogen deficiency leads to a delayed immune response allowing increased bacterial colonization resulting in persistent infection. It is known that the sustained infection results in continued inflammation that may contribute to kidney fibrosis and renal failure in the host. Thus, we conclude that physiological levels of estrogen are protective by promoting immediate robust immune responses leading to bacterial clearance and maintenance of the host immune homeostasis, following pathogen elimination.

### 9.1.3: Estrogen-mediated modulation of Dr+ *E. coli* uropathogenesis in mouse kidney is ER dependent and occurs via ERα activation.

We show that the protective effects of estrogen against Dr+ *E. coli* uropathogenesis are ER dependent and specifically involve ERα activation. Blocking of ERs with a pure ER-antagonist ICI 182, 780, in the estrogen-sufficient Non-OVX mice resulted in increased Dr+ *E. coli* colonization in their bladders and kidneys post-UTI induction. This suggested that estrogen-mediated protection against Dr+ *E. coli* in mouse urinary tract is ER dependent. Further, ICI 182,780 treatment in these mice reversed the estrogen-mediated down regulation of DAF and type IV collagen expression, suggesting that estrogen regulates DAF and type IV collagen via the classical ER dependent pathway.

The kidneys of C3H/HeJ and B6.129 mice do express both ERα and ERβ, however ERα expression is predominant. Thus, we proposed that ERα may be the functional receptor for estrogen mediated protective effects in the mouse kidney. Consistent with our hypothesis, we did find increased kidney bacterial colonization corresponding with increased DAF as well as type IV collagen expression in the ERα-/- mice as compared to the ERα+/+ mice. The ERα-/- mice also had increased bacterial colonization in their bladders as compared to the ERα+/+ mice. Consistent with our findings in OVX mice, the ERα-/- mice showed delayed innate inflammatory responses at initial time points and were responsive only later during the course of infection. In contrast, ERα+/+ mice showed a robust innate immune response soon after the onset of infection. Based on these observations we concluded that the protective effects of estrogen against Dr+ *E. coli* uropathogenesis are mediated via ERα.

**B) *In vitro* studies**

### 9.1.4: Estrogen differentially modulates Dr+ *E. coli* invasion in mIMCD3 cells in a dose-dependent manner.

E2 pretreatment of mIMCD3 cells, at physiological doses (1-10 nM) significantly reduced Dr+ *E. coli* internalization compared to that in the untreated control cells. Treatment of cells with a pharmacological dose of E2 (100 nM) prior to infection resulted in marked increase in the bacterial invasion compared to the cells treated with physiological doses of E2. These observations led us to conclude that estrogen at a physiological dose provides protection against bacterial internalization in mIMCD3 cells, whereas estrogen deficiency or a pharmacological estrogen dose induces increase in bacterial invasion in these cells.

### 9.1.5: Estrogen differentially regulates expression of DAF and induction of the tyrosine kinase activation pathway in a dose-dependent manner.

DAF expression was found to be significantly reduced in the cells treated with physiological doses of E2 compared to that in the untreated control cells and in the cells treated with a pharmacological dose of E2. Also, dose-dependent E2 regulated changes in DAF expression resulted in differential invasion of the Dr+ *E. coli* in mIMCD3 cells. We conclude that physiological levels of estrogen down-regulates DAF expression in mIMCD3 cells thus lowering bacterial invasion, while estrogen-deficiency or higher estrogen levels increase DAF expression leading to an increased bacterial invasion in the cells.

The tyrosine kinase induced PI3K/Akt signaling pathway was found to be activated during Dr+ *E. coli* internalization in mIMCD3 cells. Thus, tyrosine kinase mediated PI3K/Akt signaling activation, may represent one of the host cell signal transduction pathway during Dr+ *E. coli* internalization in these cells. Furthermore, treatment of cells with E2 at a physiological dose was found to reduce the induction of the PI3K/Akt signaling pathway, whereas treatment with a pharmacological dose of E2 significantly enhanced the PI3K/Akt pathway activation. It appears that modulation of DAF as well as tyrosine kinase activity following bacterial adherence may represent an important requirement for Dr+ *E. coli* internalization in mIMCD3 cells.

### 9.1.6: E2-mediated protective effects against Dr+ *E. coli* invasion in mIMCD3 cells occurs primarily via ERα activation pathway.

We found that blocking of ER activity by pure ER-antagonist ICI, 182 780 treatment resulted in increased Dr+ *E. coli* invasion in mIMCD3 cells. ICI, 182 780 significantly reversed the protective effects of 10 nM E2 in a dose dependent manner, when cells were co treated with E2 (10 nM) and various doses of ICI,182 780. This suggested that protective effects of E2 are ER dependent. Furthermore, we found significant reduction in bacterial invasion in the cells treated with the ERα specific agonist similar to E2 action, while ERα specific antagonist reversed the E2-mediated protection. We did not observe any effect of ERβ activation or inactivation on Dr+ *E. coli* invasion in mIMCD3. Thus, the ERα but not the ERβ pathway is involved in the E2-mediated protection against

Dr+ *E. coli* invasion in the mIMCD3 cells. In addition, ERα is the predominant subtype expressed at mRNA levels in mIMCD3 cells, explaining the functional role of ERα over ERβ in these cells. Blocking ER activity in the cells with ICI, 182,780 caused increase in Dr+ *E. coli* invasion and DAF expression compared to the cells treated with E2. Furthermore, ERα-specific activation with PPT in the infected cells induced significant reduction in DAF expression and bacterial colonization which was comparable to those in the cells treated with E2, but was significantly lower than those in the untreated control cells. These observations suggest that E2 regulated DAF expression via ERα activation serves as one of the possible mechanisms involved in estrogenic protection against Dr+ *E. coli* invasion in mIMCD3 cells.

Finally, we conclude that host endogenous levels of estrogen and ER subtype expression may play an important role in determining the host's susceptibility to UTI by Dr+ *E. coli*. A Physiological level of estrogen via activation of ERα seems to provide protection against Dr+ *E. coli* colonization by regulating expression of colonization receptors and induction of tyrosine kinase cellular signaling pathway. In addition, a physiological level of estrogen may also regulate infection in the host by generating robust early induction of innate inflammatory response in the urinary tract, allowing rapid clearance of bacteria and establishment of immune homeostasis following bacterial elimination.

## 9.2 Significance and Future directions

UTI remains a problem of clinical and medical significance due to the ever increasing rate of antibiotic resistance in UPEC and the unavailability of vaccines. This project will be the basis future studies investigating various host factors that may be responsible for occurrence of recurrent UTIs. Increased understanding of uropathogenic bacterial virulence and host susceptibility factors will allow individualization of diagnosis and therapy for UTI; this will help combat the present limitations associated with available treatment options for UTIs (Godaly & Svanborg, 2007). Furthermore, understanding of molecular mechanisms involved in bacterial pathogenesis and inflammation at the cellular level is vital for the development of novel strategies for prevention and treatment of UTIs (Sivick & Mobley, 2010).

Our study provides the first *in vivo* and *in vitro* evidences for the protective effects of a physiological dose of estrogen against Dr+ *E. coli* colonization and invasion in the kidney. Using ERα-/- mice, we report for the first time that estrogen protection against uropathogenesis is mediated via ERα. In our study, we also show that estrogen via ERα regulates expression of adherence receptors, DAF and type IV collagen, thereby regulating the attachment of Dr+ *E. coli* to the host urinary tract. Clinical studies in patients have also indicated that increase in density of UPEC epithelial receptors tend to increase their risk to have recurrent UTIs and pyelonephritis (Herrmann *et al*, 2002). Our experimental results do support the observations made in clinical settings on hormonal regulation of UTI susceptibility in patients. For instance, increase in UTI susceptibility is observed in pregnant women that exhibit high estrogen levels or in postmenopausal

women that exhibit low or deficient estrogen levels (Hu *et al*, 2004, Maloney, 2002, Mittal & Wing, 2005, Schnarr & Smaill, 2008).

Based on these observations and utilizing the available information in the literature, mechanisms for interrupting host receptor and bacterial ligand interactions can be developed as a therapeutic strategy for blocking the adherence of UPEC to host cells and thus disrupting the cycle of re-infection during uropathogenesis. Studies investigating the molecular mechanisms of UPEC colonization and invasion at cellular levels using an *in vitro* model will guide the identification of additional pharmacological regulators acting similar to physiological estrogen in regulating colonization receptor expression or in boosting innate inflammatory response in the host infected with UPEC. Based on our results, preclinical studies are needed to include prophylactic estrogen replacement therapy at physiological doses or treatment with specific ERα selective ligands. In addition, research needs to be conducted towards development of mucosal vaccines for Dr+ *E. coli* to generate local antibody responses to block UPEC binding receptors. Such vaccines will block binding to DAF and prevent Dr+ *E. coli* colonization and infection in the urinary tract of patients with recurrent infections.

In our study ERα-/- mice showed increased UTI susceptibility and this suggests that clinical studies should be conducted to look for possible reduced ERα expression or ERα polymorphism in patients with recurrent UTIs or pyelonephritis.

Our future work will focus on identifying different signaling mechanisms associated with estrogen-mediated modulation of DAF and type IV collagen utilizing *in vivo* and *in vitro* experimental models. We will also work towards studying estrogen mediated differential regulation of inflammatory responses against UPEC under different clinical settings involving patients with different estrogen levels. RNAi strategies against ERs and colonization factors will be employed under *in vitro* and *in vivo* settings to investigate the functional role of ERs and colonization receptors during UTI pathogenesis. Role of ERα and ERβ and possible ER gene polymorphism in UTI susceptible hosts will be studied. The information obtained will further guide us in identification of novel biomarkers or therapeutic strategies for treatment of UTI.

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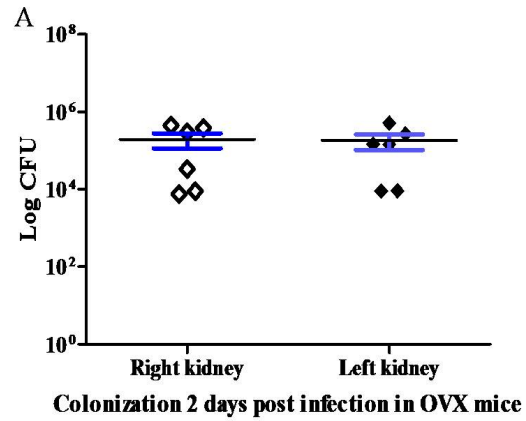
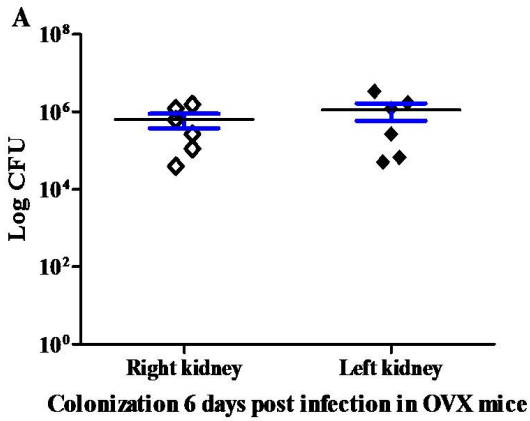
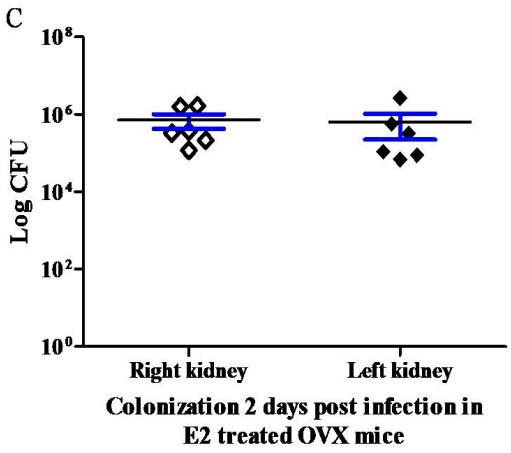
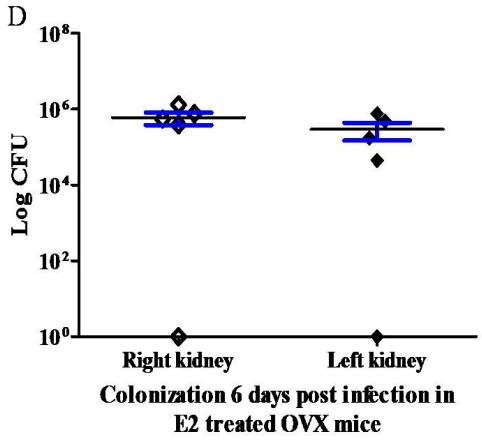
# APPENDIX 1

## Supplemental Figures

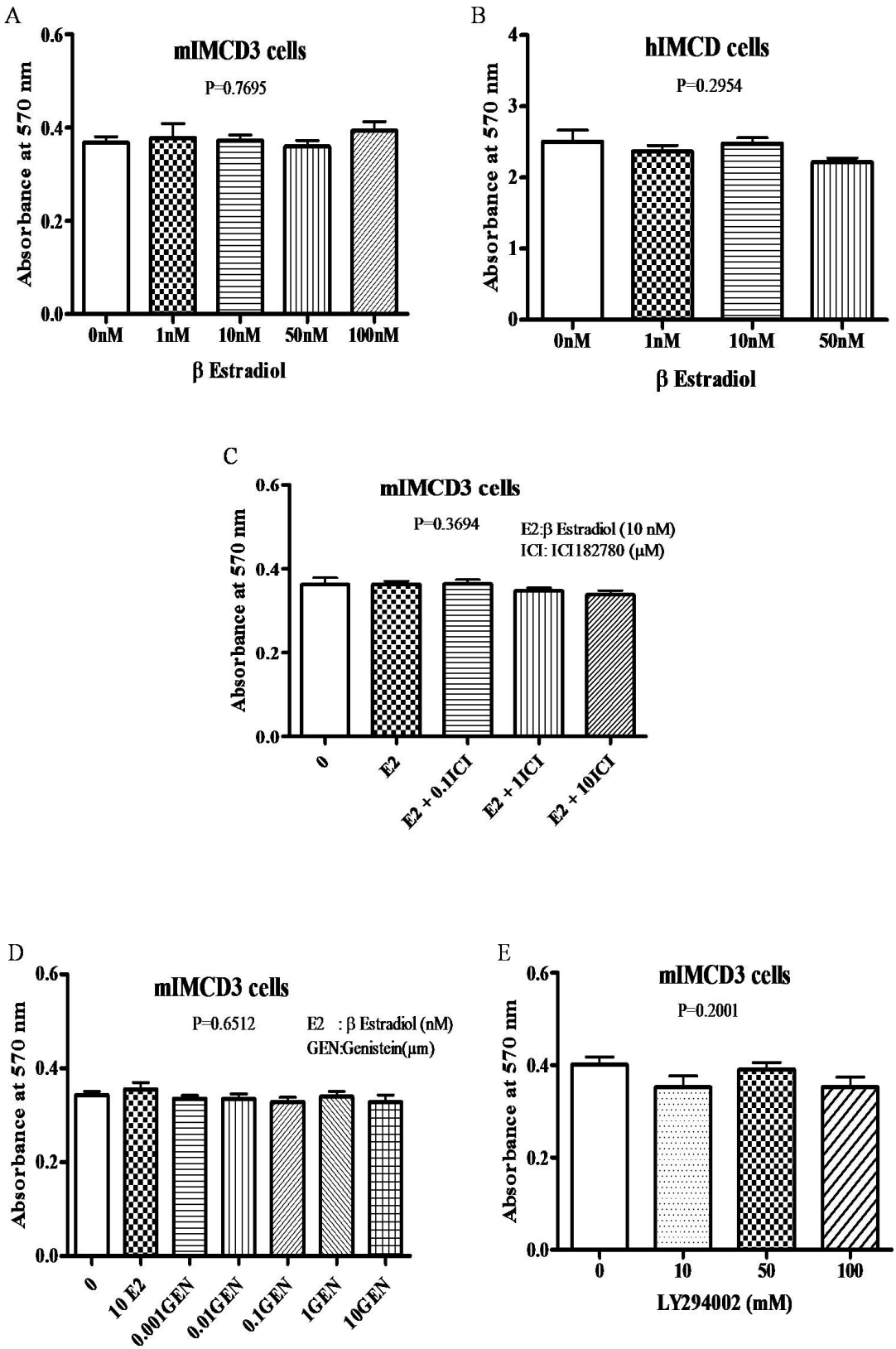


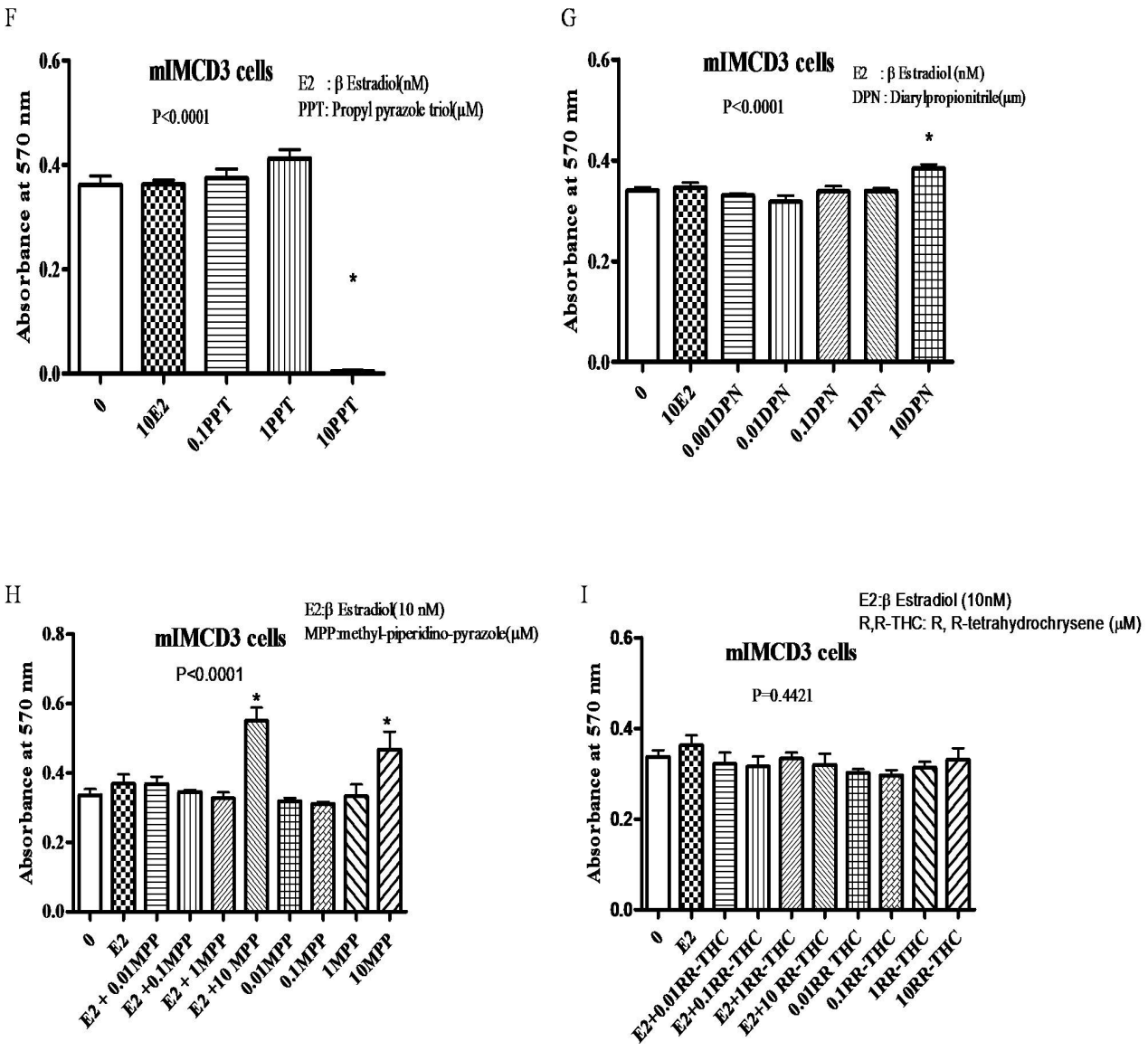



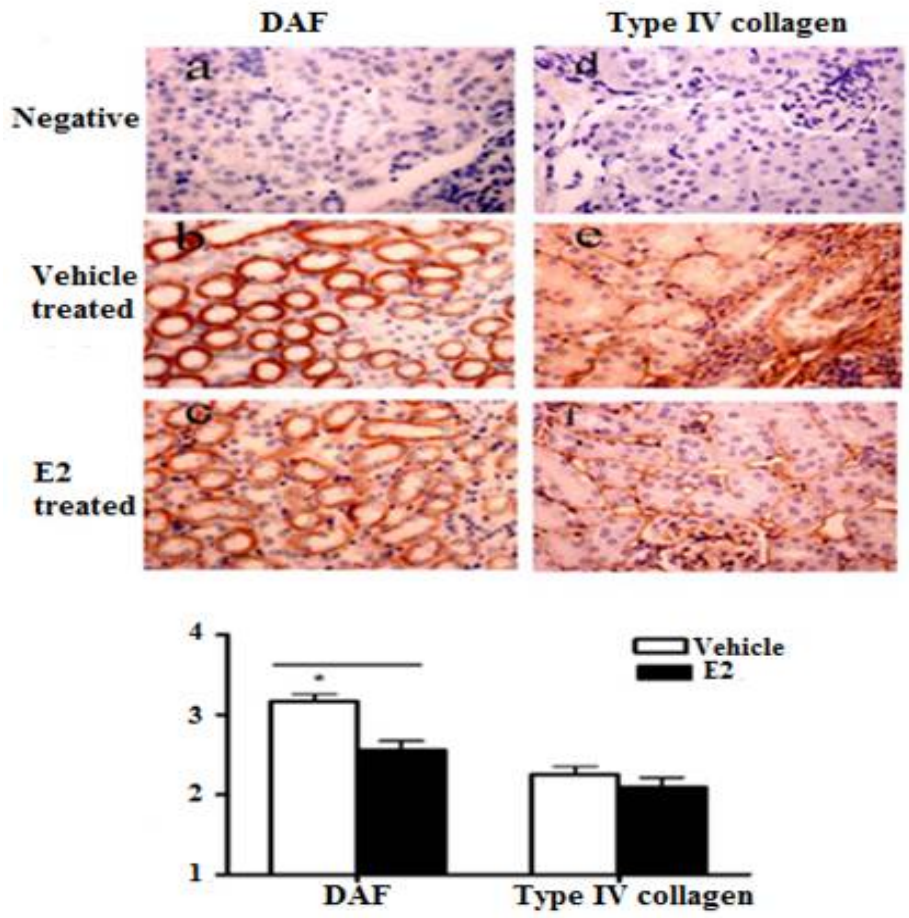




**Appendix Figure 1: Bacterial counts in the right and left kidneys of OVX C3H/HeJ mice.** Bacterial CFU per gram kidney tissue in vehicle **(A and B)** OVX and E2 **(C and D)** treated OVX mice at 2 and 6 days post-UTI induction with Dr+ *E. coli*. Bacterial CFU counts from right kidney are depicted by open diamonds and those from left kidney are indicated by filled diamonds. Each symbol in the graph represents values from the right or left kidney of a mouse. Data in each graph shows bacterial counts with mean CFU ± SEM and error bar represents data from 4-6 mice. There was no significant difference in bacterial counts from right and left kidneys of mice from each study group. Data were analyzed by Student's t-test where $P<0.05$ was considered significant.


A
mIMCD3 cells
P=0.7695
Absorbance at 570 nm
0.6
0.4
0.2
0.0
0nM
1nM
10nM
50nM
100nM
β Estradiol
B
hIMCD cells
P=0.2954
Absorbance at 570 nm
4
3
2
1
0
0nM
1nM
10nM
50nM
β Estradiol
C
mIMCD3 cells
P=0.3694
E2:β Estradiol (10 nM)
ICI: ICI182780 (μM)
Absorbance at 570 nm
0.6
0.4
0.2
0.0
0
E2
E2 + 0.1ICI
E2 + 1ICI
E2 + 10ICI
D
mIMCD3 cells
P=0.6512
E2 : β Estradiol (nM)
GEN:Genistein(μm)
Absorbance at 570 nm
0.6
0.4
0.2
0.0
0
10 E2
0.001GEN
0.01GEN
0.1GEN
1GEN
10GEN
E
mIMCD3 cells
P=0.2001
Absorbance at 570 nm
0.6
0.4
0.2
0.0
0
10
50
100
LY294002 (mM)




**Appendix Figure 2: MTT assay for drug toxicity in the mIMCD3 cells**. Toxicity of the drugs in mIMCD3 cells (A, C-I) and hIMCD cells (B) was determined by MTT assay. Cellular growth is represented as the function of absorbance at 570nm. Absorbance values are plotted as mean absorbance ± SEM. Error bars represent data from at least three independent experiments performed in triplicate. $P < 0.05$ was considered significant. Only the non-toxic doses were used for the treatment during the invasion studies. Also, the proliferative dose of MPP (10 μM) was not used in the present study. The character * indicate significant difference from the untreated control cells.




**Appendix Figure 3: Protein expression of DAF and type IV collagen in the kidneys of C3H/HeJ mice**. A) The DAF (a, b and c) and type IV collagen (d, e and f) protein expression were determined in the paraffin embedded kidney tissue sections of vehicle and E2-treated OVX mice post 21 days of UTI induction with Dr+ *E. coli* by immunohistochemistry using specific antibodies. Negative control images represents hematoxyline-eosine staining of the tissue sections done in absence of the specific antibodies. Images a, b and c are at 20 X magnification and images d. e and F are at 40X magnification in Figure A. B) Staining intensities were quantified and are represented as mean staining score ± SEM for DAF as well as type IV collagen. Staining scores represent a scale of 0 to 4, where 0 represents no staining and 4 represents maximum staining. E2-treated OVX mice showed decreased expression of DAF as well as the type IV collagen in the kidney tubules compared to vehicle treated OVX mice. (Unpublished lab data). The character * indicate significant difference between treatment groups.

VITA

Rashmi Singh

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Doctor of Philosophy/Education

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ABSTRACT: Urinary tract infection (UTI) is a disease of medical significance. One of the important clinical effects of estrogen deficiency in postmenopausal women is their increased susceptibility to UTIs. Estrogen replacement in these women is effective in prevention of bacteriuria. However, the molecular mechanisms involved with estrogen treatment against UTIs are poorly understood. Uropathogenic Dr+ *E. coli* can cause cystitis, recurrent UTI and pyelonephritis in humans, and induce chronic pyelonephritis in C3H/HeJ mice. Dr+ *E. coli* colonize and invade the host uroepithelium by adhering to decay accelerating factor (DAF) and type IV collagen and these are known to be hormonally regulated. In the present study, we investigated the role of estrogen and estrogen receptors (ER) in modulation of Dr+ *E. coli* uropathogenesis using an *in vivo* C3H/HeJ murine model and an *in vitro* model of mouse kidney inner medullary collecting duct (mIMCD3) cells. Ovariectomy (OVX) induced menopausal mice showed increased bacterial colonization in the bladder and kidney tissues compared to the ovary intact mice after experimental induction of Dr+ *E. coli* infection. Pretreatment of the OVX mice with β-estradiol (E2) at a physiological dose reduced bacterial colonization. ER antagonist ICI 182,780 pretreatment increased bacterial colonization in the ovary intact mice, suggesting ER involvement. Furthermore, UTI induction in ERα-/- mice led to increased bacterial colonization in the bladder and kidney tissues compared to the ERα+/+ mice confirming ERα involvement. Increased kidney infection corresponded with an increase in DAF and type IV collagen expression in OVX as well as ERα-/- mice. Delayed induction of TLR2 and pro-inflammatory cytokines in the OVX and ERα-/- mice at onset of infection resulted in increased bacterial colonization compared to the control mice. Protective effects of estrogen against Dr+ *E. coli* invasion were also seen in mIMCD3 cells, where physiological levels of E2 led to a 40-50% reduction in bacterial invasion and down-regulation of DAF expression. ERα selective agonist (PPT) provided significant protection against bacterial invasion, while ICI 182, 780 blocked the E2 protection. In contrast, a higher E2 dose increased bacterial invasion and DAF expression. E2 treatment activated tyrosine kinase in a dose-dependent manner in these cells. In summary, we report for the first time that a physiological dose of estrogen via ERα action provides protection against Dr+ *E. coli* colonization in the urogenital tract by modulating binding receptors and regulating activation of innate inflammatory responses.

ADVISER'S APPROVAL: Rashmi Kaul