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Cyclic regulation of apoptotic gene expression in the mouse oviduct

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Abstract

The oviduct is a dynamic structure whose function relies upon cyclic changes in the morphology of both ciliated and secretory luminal epithelial cells. Unfortunately, infection of these epithelial cells by sexually transmitted pathogens can lead to pelvic inflammatory disease, ectopic pregnancies and infertility. The disruption of normal, cyclic apoptosis in the oviductal epithelium appears to be a causal factor of sexually-transmitted oviductal pathology and therefore, these pathways represent a potential target for diagnosis and/or therapeutic intervention. The objective of this study was to determine the normal, cyclic pattern of expression for apoptotic genes in the oviduct of the naturally cycling mouse, generating fundamental information that can be applied to the development of animal models for research and/or the identification of targets for disease intervention. Whole oviducts were collected from regular cycling mice killed at 1 pm on each day of the estrous cycle and the expression of 84 key apoptotic genes determined by targeted PCR super-array. Intact and cleaved caspases were then evaluated by western blotting. The expression of mRNA for genes classified as pro-apoptotic (*Bad, Bak1 and Bok*) and anti-apoptotic (*Bag3, Bnip2 and Xiap)* was regulated by day of the estrous cycle ($P < 0.05$). Differences in the temporal expression of several p53-related genes (*Trp53bp2, Trp53inp1 and Trp73*), those specific to the TNF superfamily (*Tnfrsf10 and Tnfsf10b*) and one caspase (*Casp14*) were also observed ($P < 0.05$). The cleaved forms of Capases-3, −6 and −12 were all detected throughout the estrous cycle. These results represent the first pathway wide analysis of apoptotic gene expression in the murine oviduct.

INTRODUCTION

Proper function of the oviduct is essential for the establishment of an unassisted pregnancy: the oviduct provides the necessary micro-environment for gamete survival, maturation, fertilization and then development of the very early embryo (reviewed in (Mastroianni, 1999). To fulfill this role, the oviduct must undergo cyclic changes involving the growth and regression of its epithelial cell layer (Abe and Oikawa 1993; Abe *et al.* 1999). Cyclic changes in the ratio of ciliated to secretory epithelial cells (Shirley and Reeder 1996), their overall height (Murray and DeSouza 1995) and secretory output (Nichol *et al.* 1992; Gardner *et al.* 1996) are all reported. As such, the oviduct is a dynamic organ, reliant on processes such as regulated cell death or apoptosis to maintain cellular homeostasis and therefore function and fertility.

The epithelial cells of the oviduct also function as a barrier to infection and disease, with exposure to an array of foreign pathogens at mating possible. Again, the process of apoptosis plays a key role in maintaining cellular homeostasis, however in this case in the

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defense against pathogenic infection (Antoni *et al.* 1995). In fact, the most common sexually-transmitted bacterial pathogens, *Chlamydia trachomatis* and *Neisseria gonorrhoea* are known to target a variety of apoptotic processes within this tissue (Fan *et al.* 1998; Binnicker *et al.* 2003). These obligate intracellular parasites manipulate signaling pathways in an intricate balance to both exploit and protect the host (Koomey 2001; Zhong 2009).

Given that regulated apoptotic processes are required for the maintenance of a normal, patent oviduct and therefore fertility, and that sexually transmitted infections are known to impair oviductal function by a targeted disruption of this same cascade, it appeared to us that specific apoptotic processes could be targeted in the future for the management of oviductal diseases, especially those induced by sexually transmitted pathogens. Unfortunately, very little information is currently available on the normal cyclic pattern of apoptotic gene expression in the oviduct, effectively hampering our ability to fully interpret the results of many studies. This, in turn, impairs our ability to translate results to the clinical setting as well as refine animal models for further research.

To address this, we designed a study that would utilize real-time PCR based super-arrays to simultaneously determine the level of expression of many genes within the apoptotic pathway. The objective of this study was to determine a profile of apoptotic genes within the oviduct that are regulated during the normal estrous cycle of the mouse, thus providing knowledge that will facilitate future research in the prevention and/or control of apoptotic-regulated oviductal dysfunction. Genes of both the intrinsic and extrinsic pathways were examined, the intrinsic pathway being activated from stressors within a cell that is destined for destruction, whereas the extrinsic pathway is invoked via external, receptor mediated signals (reviewed in Delhalle, 2003). To complement this gene expression analysis, we then evaluated the presence of both the intact and cleaved form of four of the caspases by western blotting. Activation of these caspases marks one of the final functional processes in the apoptotic pathway. Both initiator and effector caspases were detected, confirming regulated apoptosis throughout the estrous cycle in the oviduct of the mouse.

MATERIALS AND METHODS

Animals

CD1 mice were purchased from Harlan Inc. (Harlan, IN) and maintained under controlled lighting (14 h light/10 h dark) with continuous access to food and water. Animal procedures are described for each experiment below and were approved by the University of Kentucky Animal Care and Use Committee according to NIH guidelines for the ethical use of animals in research.

Staging of estrous cycles

Beginning at 6–8 weeks of age, estrous cycles were staged in CD1 females by analysis of vaginal cytology. The vagina was flushed daily, at the same time each morning, with 0.9% sodium chloride using a bent, blunted borosilicate glass pipette. To acclimatize the mice to the procedure, smears were collected for the first 7–10 days without classification. Thereafter, vaginal smears were collected in individual wells of 24-well culture plates (Costar, Corning, NY) and classified according to well established morphological guidelines (Goldman *et al.* 2007; Caligioni 2009). Smears were visualized under a Motic AE21 inverted microscope (Motic Instruments, Canada) and a digital image recorded for later reference. A total of 6 mice were killed on each day of the estrous cycle and all mice were killed at 1 pm on a designated day. Immediately before sacrifice, a final confirmatory smear was collected. Mice were allocated to a particular day only after a consistent and repeatable pattern of cycling activity was recorded. Mice were killed by asphyxiation with carbon

dioxide followed by cervical luxation and the reproductive tracts exteriorized for tissue collection. Ovaries and oviducts were retrieved, separated and snap frozen on dry ice. Blood was collected by cardiac puncture and stored overnight at 4°C before serum was harvested by centrifugation.

Concentrations of oestradiol in the serum of cycling mice

The concentration of oestradiol was determined in the serum of mice killed for the analysis of gene expression by super-array (3 mice per day). Radioimmunoassays were performed bythe University of Virginia Center for Research in ReproductionLigand Assay and Analysis Core using a Beckman Coulter antibody, as described by others (O'Brien *et al.* 2006). Because the concentration of oestradiol that was measured in samples collected on each day other than proestrus was at, or below, the listed sensitivity of this radioimmunoassay, no statistical analysis was performed and the results are not reported herein.

Expression of mRNA for Cyp17a1 and Cyp19a1 in the ovaries of cycling mice

Real-time PCR was performed to determine the level of expression of mRNA for *Cyp17a1* (cytochrome P450, family 17, subfamily a, polypeptide 1) and *Cyp19a1* (cytochrome P450, family 19, subfamily a, polypeptide 1; aromatase) in the ovaries of each mouse that was sacrificed (Figure 1). Analysis of the level of mRNA for these two steroidogenic genes was used as another means of confirmation that the estrous cycle of each mouse had been staged correctly, as described by others (Soumano *et al.* 1996; Hinshelwood *et al.* 2005). Real-time PCR was performed using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) and gene specific primer pairs (*Cyp17a1* forward 5′-TGG TCA TAT GCA TGC CAA CT-3′ and reverse 5′-GAG CGT AGA CAG ATC TCG GG-3′; *Cyp19a1* forward 5′-GTC CTG GCT ACT GTC TGG GA-3′ and reverse 5′-CAA ATG CTG CTT GAT GGA CT-3′) on a Bio-Rad IQ5 system, as described previously (Al-Alem *et al.* 2007; Bridges *et al.* 2010; Jeoung *et al.* 2010). Protocol conditions consisted of denaturation at 95 °C for 30 seconds, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec with a final dissociation (melting) curve analysis. The relative level of expression of each mRNA was standardized against L19 (forward 5′-TGG TTG GAT CCC AAT GAG AC-3′ and reverse 5′-GTC TGC CTT CAG CTT GTG GAT-3′) as a housekeeping gene and analyzed by the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen 2001).

Analysis of gene expression by real-time PCR super-array

Temporal changes in the level of expression of genes that regulate apoptotic processes was determined in whole oviducts collected from naturally cycling mice by a super-array, real-time PCR analysis. Oviducts were collected from three mice at each day of the estrous cycle and the paired oviducts from each mouse were handled as individual samples for this analysis. Total RNA was isolated from each sample using Trizol (Invitrogen, Carlsbad, CA)and then purified using RNeasy (Qiagen, Valencia, CA) following the manufacturer'sdirections. Potential genomic DNA contamination was eliminated by treatment with DNase. The mouse apoptosis RT^2 Profiler PCR Arrays and RT^2 Real-Timer SyBR Green reagent were purchased from SuperArray Bioscience Corporation (Frederick, MD). Each super-array (96-well plate) contains the primers to identify 84 key genes involved in apoptosis as well as 5 housekeeping genes. A genomic DNA contamination control, reverse transcription controls and positive PCR controls are also included. The RT^2 First strand kit (SuperArray Bioscience) contained all the reagents required to reverse transcribe the total RNA to cDNA and eliminate potential genomic DNA. Real-time PCR was then performed on a Bio-Rad IQ5 system, as described previously (Al-Alem *et al.* 2007; Bridges *et al.* 2010; Jeoung *et al.* 2010). All experimental arrays passed the designated quality controls for reproducibility, reverse transcription efficiency and (lack of) genomic

DNA contamination. The relative level of expression of each mRNA was standardized against GAPDH as a housekeeping gene and analyzed by the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen 2001).

Detection of Caspases by Western Blotting

Levels of intact and cleaved Caspases-3, −6, −9 and 12 (CASP3, CASP6, CASP9 and CASP12) were assessed in whole oviducts collected from naturally cycling mice by western blotting. Oviducts were collected from cyclic mice as described above and lysed by homogenization in radio-immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA). Again, three mice were sacrificed at each day of the estrous cycle and the oviducts of each mouse were handled as an individual sample. The concentration of protein in each sample was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) and protein (50 μg per lane) separated on 10% precast SDS polyacrylamide gels before being transferred to polyvinylidene fluoride (PVDF) membranes (both from Bio-Rad, Hercules, CA) using a semi-dry transfer. Non-specific binding sites were blocked with 5% non-fat milk at room temperature for 2 h and membranes were incubated with antibodies purchased from Cell Signaling Technology (Danvers, MA) that were generated against Capase-3 (full length, #9662 and cleaved, #9664), Caspase-6 (full length, #9762 and cleaved, #9761), Caspase-9 (full length, #9504 and cleaved, #9509) and Caspase-12 (detects full length and cleaved, #2202), each at a 1:2000 dilution, overnight at 4 °C. Each blot was later incubated with an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) polyclonal goat antibody (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) to verify equal protein loading. The immuno-reaction was detected by incubating each blot with the appropriate peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:4000; Cell Signaling Technology) or donkey anti-goat secondary antibody (1:5000; Santa Cruz) for 2 h at room temperature and images acquired using an enhanced chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany).

Statistical Analysis

All data sets were tested for homogeneity of variance and normality. If these criteria were met, the effect of day of the estrous cycle on gene expression was analyzed by one-way ANOVA followed by a post-hoc Student Newman-Keuls test when significant ($P < 0.05$). When these criteria were not met, a nonparametric Kruskal-Wallis test was utilized. These analyses were performed using SigmaStat 3.5 (SystatSoftware, Inc. Point Richmond, CA). The results presented in Supplemental Table I were obtained by analysis of sequential daily changes in gene expression by Students *t*-test. Those results are provided to grant the reader a more complete understanding of apoptotic gene expression profiles in the murine oviduct.

RESULTS

Components of the Intrinsic Pathway

The level of expression of mRNA encoding three pro-apoptotic (*Bad:* BCL2-associated agonist of cell death; *Bak1:* BCL2-antagonist/killer 1; and *Bok*: BCL2-related ovarian killer protein) and four anti-apoptotic (*Bag3:* BCL2-associated athanogene 3*; Bcl2l10:* Bcl2-like 10*; Bnip2:* BCL2/adenovirus E1B interacting protein; *Mcl1:* myeloid cell leukemia sequence 1) genes of the intrinsic apoptotic pathway is shown in Figure 2 (the anti-apoptotic *Xiap* is described later in the results). Five of these seven genes were observed to undergo a cyclic regulation in their level of gene expression with mRNA consistently higher in oviducts collected on the day of diestrus ($P < 0.05$). Temporal differences in the expression of mRNA for 3 other intrinsic genes, in this case inclusive to the p53 family, were also observed and are shown in Figure 3. Expression of mRNA for *Trp73* (transformation related

protein 73) as well as *Trp53inp1* (transformation related protein 53 inducible nuclear protein 1) and *Tr53bp2* (transformation related protein 53 binding protein 2), all pro-apoptotic genes, were transiently increased in oviducts at diestrus when compared to other days of the estrous cycle ($P < 0.05$).

Components of the Extrinsic Pathway

Consistent with our observation that several members of the intrinsic pathway of apoptosis exhibited a cyclic regulation to their gene expression, analysis of extrinsic factors uncovered temporal changes as well. The expression of mRNA for *Tnfsf10* (tumor necrosis factor (ligand) superfamily, member 10) and *Tnfrsf10b* (tumor necrosis factor (receptor) superfamily, member 10b) a ligand and receptor within the TNF family of extrinsic factors, were found to differ. Levels of mRNA for both these genes were higher at diestrus when compared to the day of estrus, 48 h later ($P < 0.05$, Figure 4). Analysis of gene expression from day to day (*t*-test; Supplemental Table 1) uncovered additional TNF family members that may warrant further investigation. Inclusive to these were mRNA encoding two receptors (*Fas*: TNF receptor superfamily member 6; and *Cd40:* Cd40 antigen) and one ligand (*Cd40lg:* Cd40 ligand). No statistically significant difference was detected in the expression of mRNA encoding *Ltbr* (lymphotoxin B receptor), *Fasl* (Fas ligand, TNF superfamily, member 6), *TNF-α* (tumor necrosis factor) or *Cd70* (Cd70 antigen).

The Caspase Cascade—No dramatic differences in the expression of mRNA for members of the caspase familly were observed during the estrous cycle. Expression of mRNA for *Casp14* (Caspase-14) was higher in oviducts collected from mice at diestrus than metestrus ($P < 0.05$, Figure 5), and the level of mRNA encoding *Xiap* (X-linked inhibitor of apoptosis), an inhibitor of Caspase-3, −7 and −9, was higher at diestrus than proestrus or estrus ($P < 0.05$, Figure 2). Cleaved CASP3, CASP6 and CASP12 were all detected by western blotting, along with their uncut precursors (Figure 6), with no dramatic temporal pattern of expression apparent. Interestingly, of the two bands (17 and 19 kDa) that were expected for cleaved CASP3, the 17 kDa band was only observed when oviducts were collected from mice sacrificed at estrus. Cleaved CASP9 was not detected by western blotting (not shown).

DISCUSSION

Sexually-transmitted pathogens such as *C. trachomatis* and *N. gonorrhoea* target apoptotic processes within the oviduct (Fan *et al.* 1998; Binnicker *et al.* 2003). In this report, we analyzed the pattern of expression of many of the genes involved in the apoptotic cascade over the natural estrous cycle of the laboratory mouse. It is our belief that by gaining an increased understanding of the normal genetic pathways involved in maintaining homeostasis and function of the oviduct, research investigating specific signaling pathways affected by infection with these bacterial agents can be designed more completely and the resultant data better extrapolated to the clinical setting. This may be especially pertinent given the increasing resistance to antibiotic therapy of infectious agents such as *N. gonorrhoea* (Tapsall *et al.* 2010; Whiley *et al.* 2010).

It is clear that sexually infectious intracellular pathogens have evolved the means to survive, utilizing us as hosts and adapting our bodies' natural apoptotic processes to their advantage. Mechanisms whereby *C. trachomatis* can block host apoptosis include inducing the degradation of pro-apoptotic proteins from the mitochondria (Fan *et al.* 1998; Dong *et al.* 2005; Ying *et al.* 2005), inhibiting antigen presentation (Zhong *et al.* 2001; Kawana *et al.* 2007) and stabilizing proteins with an inhibitory apoptotic role (Rajalingam *et al.* 2006). Infection with *N. gonorrhoea* can induce the infiltration of immune cells and stimulate

apoptosis at the epithelial cell interface (Witt *et al.* 1976). Specific membrane proteins are involved, including the bacterial PorB porin which allows a rapid calcium influx into the affected cell (Martin and Green 1995; Muller *et al.* 1999). Importantly, the regulated destruction of epithelial cells can allow pathogens access to deeper, uninfected tissues (McGee *et al.* 1981).

As expected, some commonality was identified among apoptotic genes exhibiting a cyclic pattern of expression and those targeted by apoptotic processes. For example, members of the well recognized Bcl-2 family include both pro- and anti-apoptotic members. *Bim* (BCL2-like 11, apoptosis facilitator) and *Bmf* (BCL2 modifying factor) are reported to mediate *N. gonorrhoea*-induced infection through the control of BAK1 and BAX (BCL2-associated X protein) (Kepp *et al.* 2009), and *C. trachomatis* was reported to resist apoptosis through the suppression or destruction of BIM, BAD and PUMA (BBC3, BCL2 binding component 3) proteins (Fischer *et al.* 2004). Of these, mRNA for *Bak1* and *Bad* appeared to be the most regulated within an estrous cycle. It should be noted though that matching changes in gene expression to known pathogen-affected apoptotic processes is not the objective of the study herein. Rather, our aim was to provide a more complete overview of apoptotic genes that are regulated in a temporal manner over the course of an estrous cycle in the mouse.

Among the apoptotic genes differentially expressed over time, consistency was observed in their overall manner of regulation. With increasing concentrations of circulating estradiol, mice progressed from diestrus into proestrus and the expression of all the genes we identified as cyclic-regulated within the oviduct was decreased. In contrast, as mice progressed to metestrus and diestrus, mRNA for changing apoptotic genes was uniformly increased. A general trend in the regulation of apoptotic gene expression by ovarian steroids could be hypothesized by this temporal pattern. Indeed, the regulation of oviductal function by ovarian steroids was established many years ago (Mason 1952; Lehrman and Brody 1957), epithelial localization of the respective steroid receptors is well documented (Teilmann *et al.* 2006; Shao *et al.* 2007) and both estrogen and progesterone receptor mediated signaling of apoptotic mediators have been defined (Mintz *et al.* 2008; Yoshida *et al.* 2010).

It is also likely that the heterogeneity of cells within the oviduct and our limited sample size has decreased our ability to statistically identify some differences in gene expression across the estrous cycle. A striking example of this and what appears to be a gene negatively regulated by estradiol is the mouse homolog of *Bcl2l10*. Expression of mRNA for *Bcl2l10* was ~10-fold higher in oviducts collected at estrus versus proestrus (Supplemental Table 1) and a search of the UCSC Genome Browser (Kent *et al.* 2002) indicated that there is an estrogen receptor binding site upstream of the *Bcl2l10* promoter. Overall, the trend for gene expression to be increased at diestrus, regardless of whether the apoptotic gene was pro- or anti-apoptotic in function was an unexpected finding. However, with circulating steroids at basal levels and the reproductive cycle at a relatively quiescent stage, it could be construed that this is an opportune time to repair and ready the oviduct for the next ovulation.

An evaluation of functional activation of apoptosis within the cycling oviduct was included as a final objective in this study. Caspase activation is considered one of the most specific indicators of apoptosis (Gown and Willingham 2002) and hence, caspases-3, −6, −9 and −12 (CASP3, CASP6, CASP9 and CASP12), in their full length and cleaved states, were examined by western blotting, rather than the more ubiquitous TUNEL assay that detects DNA fragmentation. Caspase-9 and −12 are classed as initiator or pro-apoptotic caspases, whereas Caspases-3 and −6 are classed as downstream effector caspases that are cleaved and activated by these aforementioned initiators. The full length and cleaved forms of caspases-3, −6 and −12 were detected on each day of the estrous cycle, indicating that some

level of functional apoptosis is ongoing within the oviduct throughout the entire reproductive cycle.

In summary, genes of the apoptotic pathway that are expressed within the cycling mouse oviduct show a temporal pattern in their level of expression. Genes of both the intrinsic and extrinsic pathways were readily detected and an overall trend for increased gene expression was observed at diestrus, however cleaved caspases signifying functional apoptosis could be detected on each day of the estrous cycle. With sexually-induced pathogens known to target apoptotic processes within the oviduct, it is hoped that a better understanding of the normal pattern of apoptotic gene expression will aid in the further study, diagnosis and or therapeutic intervention of apoptosis-related oviductal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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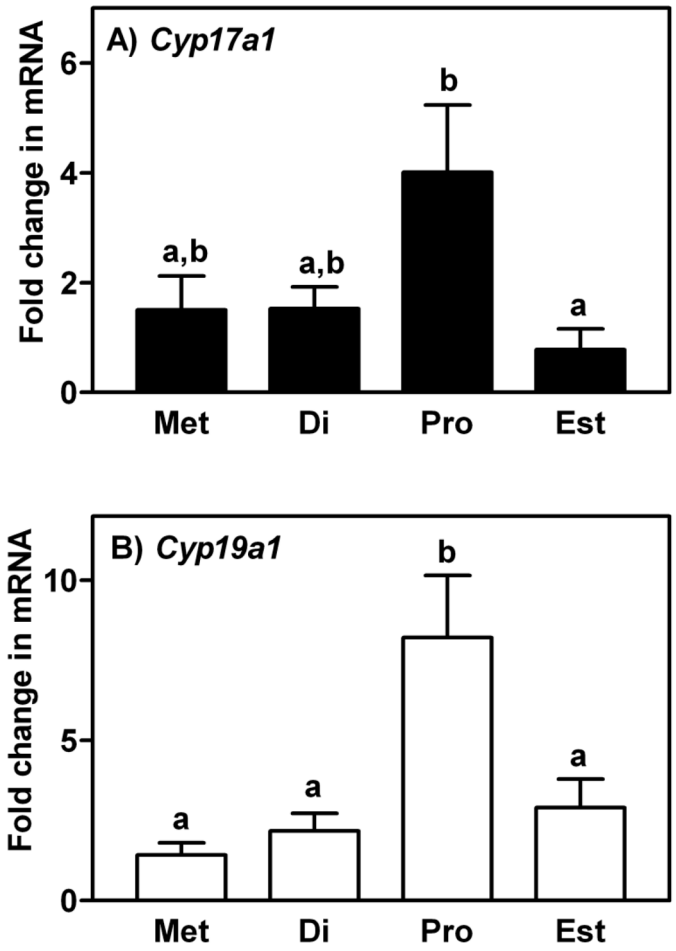

Figure 1.

Expression of mRNA encoding A) *Cyp17a1* and B) *Cyp19a1* in the ovaries of mice sacrificed at each day of the estrous cycle. Data are the means ± SEM of three samples per day. Levels of mRNA were obtained by real-time PCR, expressed as fold changes and analyzed by one-way ANOVA. For each mRNA within a panel, values with different superscript letters differ ($P < 0.05$).

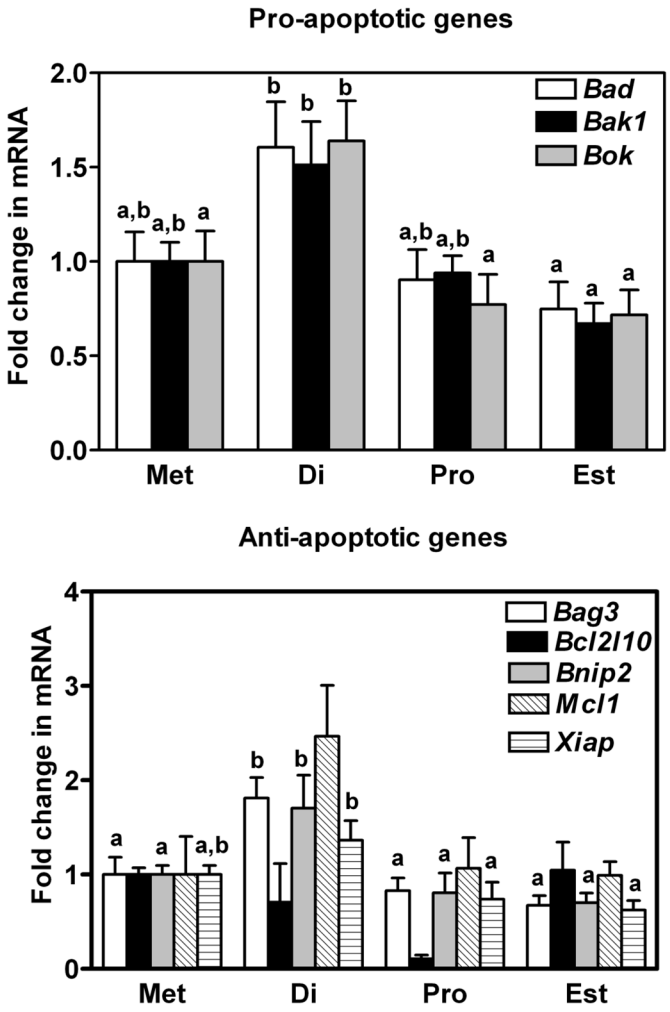

Figure 2.

Expression of mRNA encoding A) *Bad*, *Bak1* and *Bok*, and B) *Bag3*, *Bcl2l10*, *Bnip2*, *Mcl1* and *Xiap* in the oviducts of mice sacrificed at each day of the estrous cycle. Data are the means ± SEM of three samples per day. Levels of mRNA were obtained using mouse apoptosis RT^2 Profiler PCR Arrays by the $2^{-\Delta\Delta}$CT method. Data was analyzed by one-way ANOVA. For each mRNA within a panel, values with different superscript letters differ ($P < 0.05$).

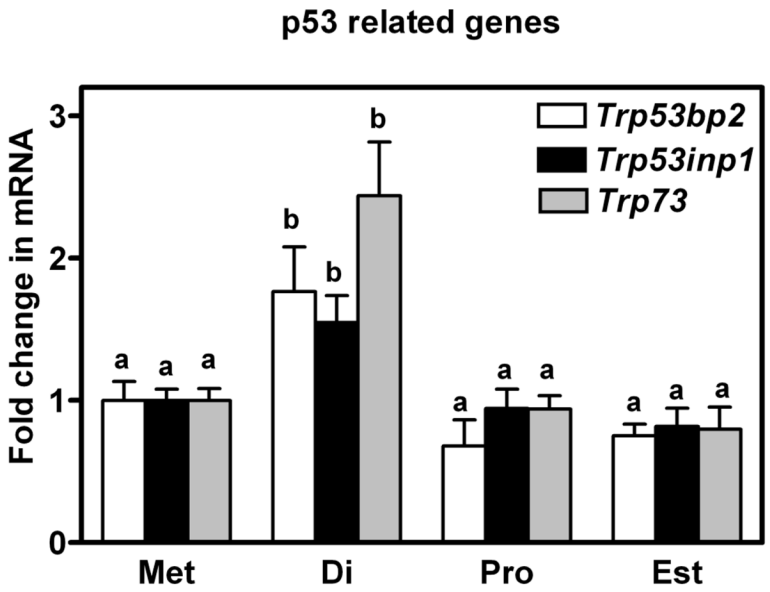

Figure 3.

Expression of mRNA encoding *Trp53bp2*, *Trp53inp1* and *Trp73* in the oviducts of mice sacrificed at each day of the estrous cycle. Data are the means ± SEM of three samples per day. Levels of mRNA were obtained using mouse apoptosis RT^2 Profiler PCR Arrays by the $2^{-\Delta\Delta}$CT method. Data was analyzed by one-way ANOVA. For each mRNA, values with different superscript letters differ ($P < 0.05$).

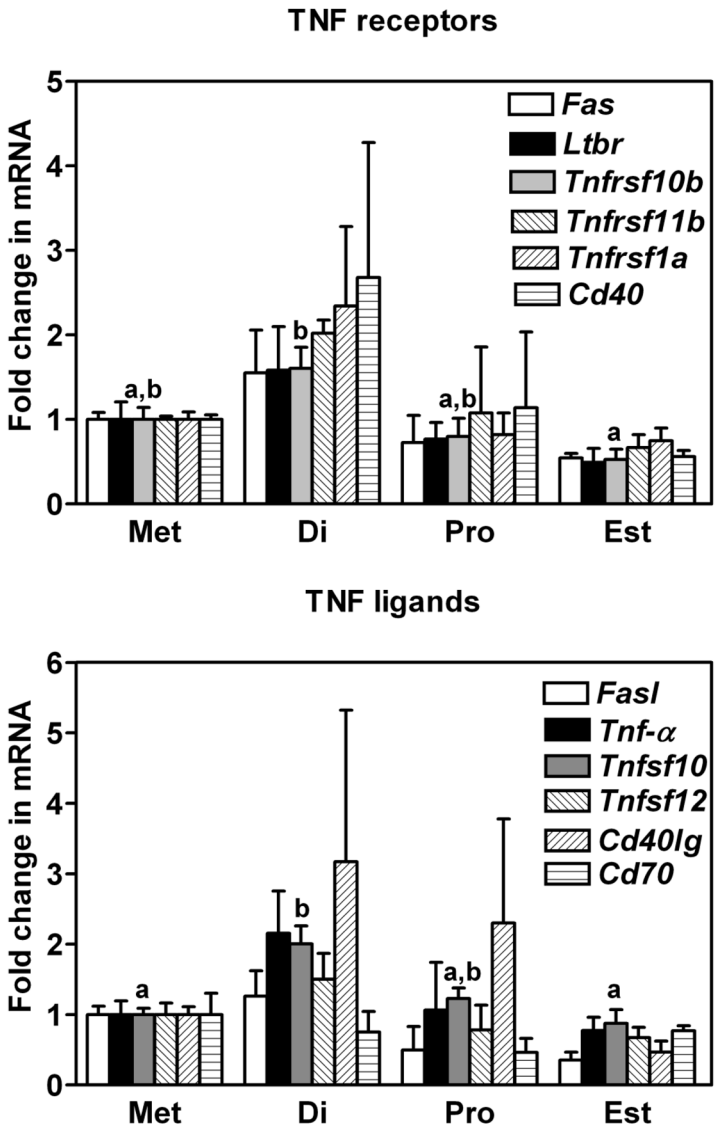

Figure 4.

Expression of mRNA encoding A) *Fas, Ltbr, Tnfrsf10b, Tnfrsf11b, Tnfrsf1a* and *Cd40* and B) *Fasl, Tnf-α, Tnfsf10, Tnfsf12, Cd40lg* and Cd70 in the oviducts of mice sacrificed at each day of the estrous cycle. Data are the means ± SEM of three samples per day. Levels of mRNA were obtained using mouse apoptosis RT^2 Profiler PCR Arrays by the $2^{-\Delta\Delta}$CT method. Data was analyzed by one-way ANOVA. For each mRNA within a panel, values with different superscript letters differ ($P < 0.05$).

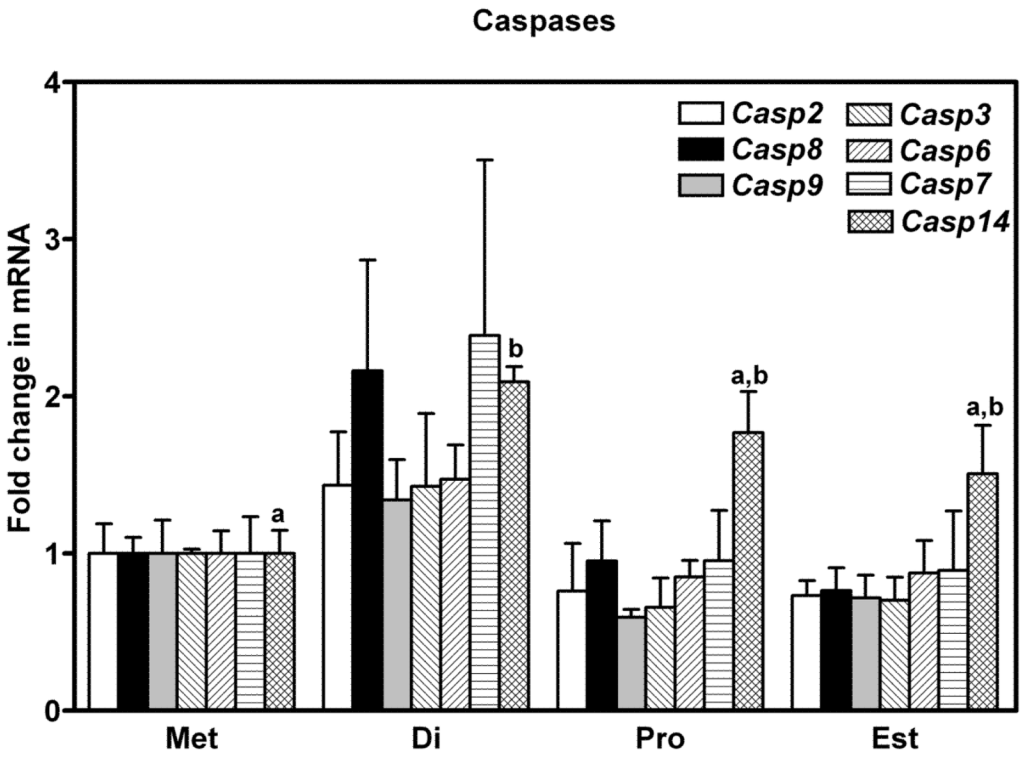

Figure 5.

Expression of mRNA encoding *Caspases 2, 3, 6, 7, 8, 9* and *14* in the oviducts of mice sacrificed at each day of the estrous cycle. Data are the means ± SEM of three samples per day. Levels of mRNA were obtained using mouse apoptosis RT^2 Profiler PCR Arrays by the $2^{-\Delta\Delta}$CT method. Data was analyzed by one way ANOVA. For each mRNA, values with different superscript letters differ ($P < 0.05$).

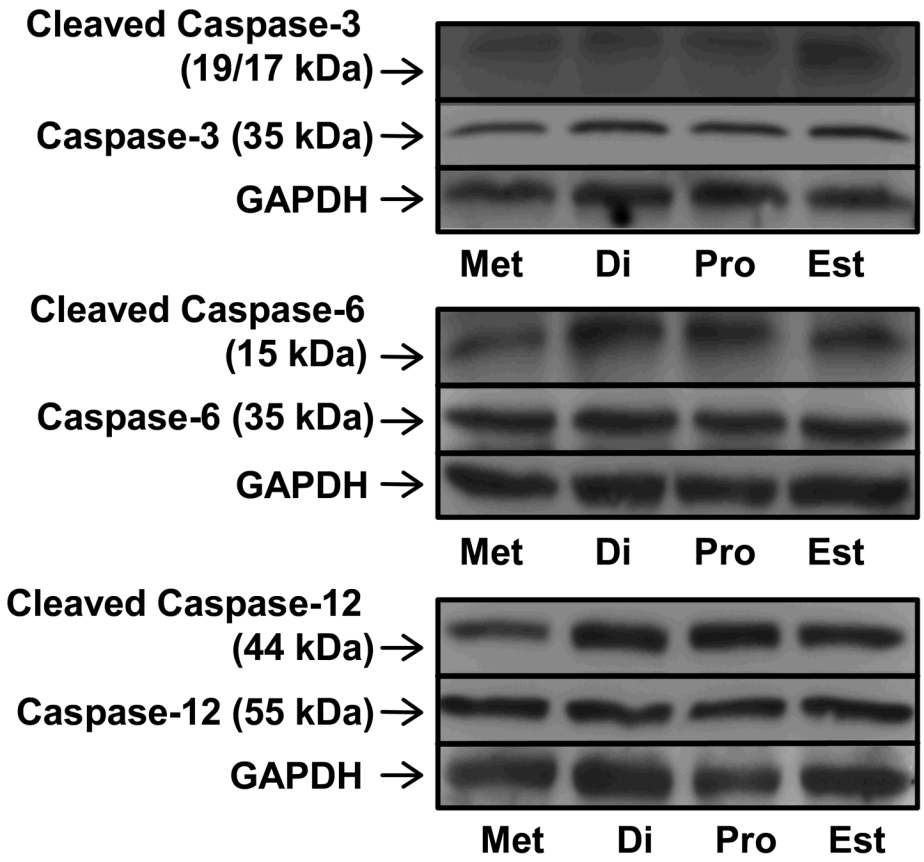

Figure 6.
Detection of full length and cleaved CASP3, CASP6 and CASP12 in the oviducts of mice sacrificed at each day of the estrous cycle. The images are representative of three western blots (replicates) performed for each caspase with GAPDH used as a loading control.