Influence of 17β-Estradiol on Gene Expression of *Paracoccidioides* during Mycelia-to-Yeast Transition

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### Abstract

**Background:** *Paracoccidioides* is the causative agent of paracoccidioidomycosis, a systemic mycosis endemic to Latin America. Infection is initiated by inhalation of conidia (C) or mycelial (M) fragments, which subsequently differentiate into yeast (Y). Epidemiological studies show a striking predominance of paracoccidioidomycosis in adult men compared to premenopausal women. In vitro and in vivo studies suggest that the female hormone (17β-estradiol, E₂) regulates or inhibits M-or-C-to-Y transition. In this study we have profiled transcript expression to understand the molecular mechanism of how E₂ inhibits M-to-Y transition.

**Methodology:** We assessed temporal gene expression in strain Pb01 in the presence or absence of E₂ at various time points through 9 days of the M-to-Y transition using an 11,000 element random-shear genomic DNA microarray and verified the results using quantitative real time-PCR. E₂-regulated clones were sequenced to identify genes and biological function.

**Principal Findings:** E₂-treatment affected gene expression of 550 array elements, with 331 showing up-regulation and 219 showing down-regulation at one or more time points (p≤0.001). Genes with low expression after 4 or 12 h exposure to E₂ belonged to pathways involved in heat shock response (hsp90 and hsp70), energy metabolism, and several retrotransposable elements. Y-related genes, α-1,3-glucan synthase, mannosyltransferase and Y20, demonstrated low or delayed expression in E₂-treated cultures. Genes potentially involved in signaling, such as palmitoyltransferase (erf2), small GTPase RhoA, phosphatidylinositol-4-kinase, and protein kinase (serine/threonine) showed low expression in the presence of E₂, whereas a gene encoding for an arrestin domain-containing protein showed high expression. Genes related to ubiquitin-mediated protein degradation, and oxidative stress response genes were up-regulated by E₂.

**Conclusion:** This study characterizes the effect of E₂ at the molecular level on the inhibition of the M-to-Y transition and is indicative that the inhibitory actions of E₂ may be working through signaling genes that regulate dimorphism.

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**Introduction**

*Paracoccidioides* is a thermally dimorphic fungus that is the etiological agent of paracoccidioidomycosis (PCM) [1]. Until recently, *P. brasiliensis* has been considered the only species in this genus. However, assessment of the molecular, phylogenetic analysis, and morphological characteristics of numerous isolates of *Paracoccidioides* has resulted in the differentiation of the genus into two species, *P. brasiliensis* and *P. lutzii*, the latter recently proposed as the new species comprised of the Pb01-like isolates [2]. PCM, endemic to Central and South America, is one of the most common systemic mycoses in these areas with a striking predominance of clinical disease in adult men [3, 4]. Infection usually occurs after inhalation of the saprophytic form conidia (C) or mycelial (M) propagules, which reach the alveolar epithelium, where they transform into the parasitic yeast (Y) form. The temperature of 37°C triggers its dimorphism in the host or in culture, which results in changes in cellular and cell wall constituents [5, 6]. Genomic approaches, such as EST (expressed sequence tag) analysis or suppression subtraction hybridization, and cDNA or gDNA microarrays have been used to identify differentially expressed genes in each morphological phase, during M-or-C-to-Y transition, or during host-pathogen interaction [7–19]. These studies have highlighted how this fungus adapts to its environment.

The morphological differentiation that takes place during M-or-C-to-Y transition is considered vital in the pathogenesis of PCM; strains that are unable to transform into yeasts are not virulent.
Epidemiological studies of PCM patients indicate that the disease is about 11 to 30 times more common in adult males despite equal frequencies of exposure to this fungus by both genders [4,22–28]. Furthermore, clinical disease in prepubertal or postmenopausal females is equal to that in males [4]. This epidemiological feature of PCM led to the hypothesis of a steroidal interaction between mammalian hormones and this organism. In vivo studies have shown that E2 inhibits the M-or-C-to-Y transition [29,30]. Additional studies have shown high affinity steroidal-specific binding of E2 to a cytosolic protein from mycelia or yeast [31,32]. Furthermore, blocking of dimorphism was demonstrated in animal studies [33,34]. Since many fungi interact with pheromones or hormones, which modulate fungal behavior [35], fungal genes responsive to hormones are of great interest and possess potential clinical relevance. Thus, our aim in this study was to examine the influence of E2 on gene expression by *Paracoccidioides* during thermally induced morphological transition from the M to the Y-form, and determine which, if any, genes might show an altered expression in the presence of E2. Our results indicate changes in the expression of various genes in different functional categories.

**Results**

The goal of our study was to delineate the effects of E2 on *Paracoccidioides* while the organism was undergoing its thermally-induced form transition from the infective M-form to the Y-form found in infected tissues. We approached this goal by first examining the morphologic transition to confirm that E2 was inhibiting it, and then sampling the fungal mass from those same cultures to develop a transcriptional profile of the organism during form transition.

**E2 effects**

**Effects on M-to-Y transition.** Following the temperature switch of a mycelia culture from 25°C to 37°C, hyphae were maintained morphologically through the 12 h time point, and were indistinguishable among controls or E2-treated cultures. At the 24 h time point, prominent changes in the hyphal morphology, characterized by intercalary or lateral swellings, were observed in controls, but not the E2-treated cultures. At the 72 h time point, we observed more transforming hyphae in the controls than in E2-treated samples. Transformation into yeast, characterized by protrusion of buds or multiple buds from chlamydospores, was observed at 144 h. Free mature, multiply budding yeast were observed after 216 h in the control samples. In contrast, hyphae were maintained in E2-treated samples until the 144 h and 216 h time points, with only rare cells in hyphae showing a rounding or progression through transition into a Y-form (Figure 1A). These data are indicative that Pb01 responds functionally to the presence of E2 (i.e., E2 inhibition of M-to-Y transition) in the same manner as did *P. brasiliensis* in our previous studies [29,36,37]. This validates our use of the Pb01 strain for the current experiments.

**E2 effects on transcriptional profile.** We assessed gene expression of Pb01 during M-to-Y transition at various times through 216 h of transition by evaluating transcripts for changes in expression in the presence or absence of E2. We employed a random-shear genomic DNA microarray containing over 11,000 elements, previously developed in our laboratory [8]. In comparison with controls, E2 treatment significantly altered transcript levels (using a p-value cutoff of 1e-3) in genes represented by 550 array elements, with 331 of the elements showing upregulation and 219 showing down-regulation at one or more time points (Figure 1B).

We felt that gene expression during the earlier stages of M-to-Y transition might be the most important in regulating the change in morphology. Thus, we sequenced and annotated 92 array elements showing significantly altered transcript levels at earlier time points (i.e., 4, 12, or 24 h) in E2-treated in comparison to controls. In addition, we searched the previously sequenced array elements [8] for changes in transcript expression and compiled the results. From 916 sequenced array elements, 164 showed altered mRNA expression in the presence of E2 at one or more time points. However, 21 and 29 sequenced elements showed nonspecific and no hits in the database, respectively, and were excluded from the analysis. Subsequent analysis showed that 47 array elements could be assigned to a functional category. These are listed, with their expression values, in Table 1 and 2; 33 elements showing sequence similarity only with hypothetical or predicted proteins and 3 with ESTs of Pb01 with unknown function are excluded from the table. The annotated function of the 47 genes included a variety of categories, such as energy metabolism, cell structure and morphology, transporters, heat-shock response, oxidative stress response, ribosomal proteins, ubiquitin-mediated protein degradation, signal transduction, RNA processing, chromatin structure, and others. We also found 31 array elements that had sequence similarity with retrotransposon elements. A heat map of temporal transcript expression of 47 functional category genes and 31 retrotransposon array elements in the presence of E2 in comparison to the transcript expression of the pooled controls during form-transition is presented in figures 2A and 2B, respectively.

**Quantitative real-time PCR expression analysis of selected genes**

Confirmation of gene expression analysis of selected genes was carried out by using quantitative real-time PCR (qRT-PCR). We chose to examine some genes on the basis of their known expression patterns during form-transition to follow morphogenesis and confirm that the control cultures were progressing through morphogenesis as expected. Other genes were chosen on the basis of significant changes shown on the microarrays, or because of their potential to be involved in E2-mediated changes in transcription. The genes chosen for the qRT-PCR studies included those encoding heat-shock proteins (i.e., hsp90, hsp70, hsp40), hydrophobin, flavodoxin (I20), chitin synthase (chs4), glucosamine-fructose-6-phospho aminotransferase (gfa1), β-1,3-glanucan synthase (gls1), α,α,1,3-glanucan synthase (mok11), GTPase RhoA, phosphatidlyinositol 4-kinositol 4-kinase, ATP binding cassette-cdr4 (cdr4), β-tubulin, ribosomal protein-B35 (B35), E3 ubiquitin-protein ligase (ubr111) and a retrotransposon element (repepsin). All were examined during the temporal event of M-to-Y transition. Not all genes chosen for the qRT-PCR studies are known to be on the microarray, e.g., mok11 and hsp90. Hydrophobin, mok11, gfa1, chs4, gls1 and I20 were selected for qRT-PCR because of their known expression patterns, and their involvement in the structure and maintenance of the cell wall [8,38]. Heat-shock proteins (i.e., hsp90, hsp70 and hsp40) are well studied [39–41]. cdr4 and hsp90 were selected because cdr1, cdr2 and hsp90 in *Candida albicans* have been shown to respond to E2 [42]. Phosphatidylinositol 4-kinase and GTPase RhoA were chosen because they are involved in signal transduction [9]. β-tubulin and B35 were chosen as control genes and have been used previously [8]. Details of the primers used for each of these genes are listed in Table 3.

Figures 3A-N show β-tubulin normalized relative mRNA levels of each gene determined by qPCR; data from B35 and β-tubulin controls are not included (data not shown). The correlation coefficient of qRT-PCR results and microarray data was positive.
for 11 of the 14 functional category genes examined. Different normalization steps used for qRT-PCR and microarray likely resulted in discrepancies in the correlation of expression determined by microarray versus that found by qRT-PCR.

**Heat-shock response.** Previous studies with *Paracoccidioides* have shown that *hsp90* or *hsp70* are induced during M-to-Y transition and are crucial for its adaptation to increased temperature [11,40,43]. In the present study, *hsp90*, *hsp70* and *hsp40* showed differentially regulated transcription by qRT-PCR in response to E2. *hsp90* and *hsp70* showed low numbers of transcripts at initial time points (4 or 12 h) during transition in response to E2 (Figures 3A and 3B). In contrast, *hsp40/DnaJ*
showed high transcript numbers at the initial time points (4 and 12 h) in response to E2 (Figure 3C). However, we observed a significant drop in transcript numbers for *hsp90* 4 or 12 h in controls compared to E2-treated samples in comparison to controls (Figure 3A–C); a significant drop occurred for only for *hsp70* or *hsp40* at later time point (i.e., 0 h) in E2-treated samples (Figure 3I). The increased mRNA expression was observed in both control and E2-treated samples (Figure 3D). As anticipated, in comparison with the baseline 0 h time point, significant upregulation of transcripts of the flavodoxin-1,3-glucan synthase, chitin synthase, glucosamine-fructose-6-phospho amidotransferase and β-1,3-gluca synthase occurred at 4 or 12 h in controls compared to E2-treated samples (Figures 3E–H). Thus, control cultures behaved as expected, with decline of transcripts of an M-related gene and increased transcription of genes associated with M-to-Y transition and the Y form.

The primary polysaccharide synthase of the yeast cell wall, α-1,3-glucan synthase (Y-related gene) in the control cultures indicated 1,3-glucan synthase was observed for E2-treated samples at 4 or 12 h. Therefore, to understand how the presence of E2 affects transcription of genes related to the M-to-Y morphogenesis of Pb01, we performed qRT-PCR for M- or Y-form related or specific genes. The expression of the M-form related gene, hydrophobin, was maintained in control and E2-treated samples until 72 h compared to the baseline 0 h time point (Figure 3D). However, somewhat higher mRNA expression was seen for hydrophobin in E2-treated samples in comparison to control during the initial 24 h of transition; at later times (144 and 216 h) a significant drop in expression was observed in both control and E2-treated samples (Figure 3D).

### Table 1. Sequence analysis of E2 regulated gene and expression values in comparison to controls at various time-points during M-to-Y transition.

| Gene product | Accession number (GI) | BLASTX (Fungi); E-value | M | E2-Control (Ratio) |
|--------------|-----------------------|-------------------------|---|------------------|
|              |                       |                         | 0 | 4               |
|              |                       |                         | 12| 24              |
|              |                       |                         | 72| 144             |
|              |                       |                         | 216|                |
| 1 | Isocitrate lyase ‘icl’ (JM_27g7) | 226280967 3e-34 | 2.85 | 0.06 |
| 2 | ATP synthase F0 subunit-9 (JM_82c3) | 68302975 2e-12 | 0.005 | 0.09 |
| 3 | Cytochrome oxidase subunit I (JM_106a4) | 68302975 4e-72 | 0.0007 | 1.44 |
| 4 | Cytochrome oxidase subunit II (JM_122c7) | 68302975 1e-61 | 0.012 | 0.6 |
| 5 | Cytochrome c oxidase assembly protein ‘cox19’ (JM_93a2) | 226285269 6e-23 | 1.49 | 0.74 |
| 6 | NADH-dehydrogenase subunit III (JM_25g7) | 63081165 8e-006 | 0.0003 | 2.28 |
| 7 | Complex 1 protein (LYR family) (JM_20a9) | 226285694 2e-20 | 1.13 | 1.31 |
| 8 | NADH-dehydrogenase subunit II (JM_15b6) | 68302975 2e-95 | 0.0009 | 1.85 |
| 9 | NADH-dehydrogenase subunit IV (JM_129g4) | 68302875 0.0 | 0.0006 | 0.41 |
| 10 | NADH-dehydrogenase subunit V (JM_17g1) | 68302975 2e-27 | 0.0001 | 1.52 |
| 11 | NADH-ubiquinone oxidoreductase 105 kDa subunit (JM_85h6) | 226277923 9e-09 | 0.0001 | 1.42 |
| 12 | Alpha-ketoglutarate decarboxylase (JM_68e9) | 226283222 3e-55 | 0.54 | 0.84 |
| 13 | Triosephosphate isomerase ‘glycolytic enzyme’ (JM_66g5) | 226279847 1e-111 | 0.83 | 0.94 |

E2 affects cell wall and membrane-related genes during transition. Dimorphic transition (i.e., M-to-Y) causes changes in the composition of cell wall components and plasma membrane lipids [6]. Therefore, to understand how the presence of E2 affects transcription of genes related to the M-to-Y morphogenesis of Pb01, we performed qRT-PCR for M- or Y-form related or specific genes. Expression of the M-form related gene, hydrophobin, was maintained in control and E2-treated samples until 72 h compared to the baseline 0 h time point (Figure 3D). However, somewhat higher mRNA expression was seen for hydrophobin in E2-treated samples in comparison to control during the initial 24 h of transition; at later times (144 and 216 h) a significant drop in expression was observed in both control and E2-treated samples (Figure 3D). As anticipated, in comparison with the baseline 0 h time point, significant upregulation of transcripts of the flavodoxin-1,3-glucan synthase, chitin synthase, glucosamine-fructose-6-phospho amidotransferase and β-1,3-gluca synthase occurred at 4 or 12 h in controls compared to E2-treated samples (Figures 3E–H). Thus, control cultures behaved as expected, with decline of transcripts of an M-related gene and increased transcription of genes associated with M-to-Y transition and the Y form.

The primary polysaccharide synthase of the yeasts cell wall, α-1,3-glucan synthase (Y-related gene), showed significant upregulation of transcripts in control and E2-treated samples until 72 h compared to the baseline 0 h time point, but showed significantly reduced transcript expression in E2-treated samples at later time points (144 and 216 h) (Figure 3D). The increased mRNA expression α-1,3-glucan synthase in the control cultures indicated a change to the Y-form that was corroborated by the observed morphology (Figure 1A). In contrast, a significant decrease in mRNA expression of α-1,3-glucan synthase was observed for E2-treated samples.
treated cells from 72 h onwards, which correlated with the observed lack of significant morphological changes in those cultures.

Signaling genes potentially involved in M-to-Y transition

Genes such as palmitoyltransferase ‘erf2’, small GTPase RhoA, arrestin domain-containing protein, phosphatidylinositol 4-kinase, serine/threonine-protein kinase are related to G-protein mediated signaling and the phosphatidylinositol signaling system. By microarray all but the arrestin domain-containing protein showed lower transcript expression in E2-treated cultures at the initial time points (4 or 12 h) compared to controls (Figure 2A). In contrast, the gene encoding for an arrestin domain-containing protein showed higher transcript expression on the microarray (Figure 2A). qRT-PCR data for GTPase RhoA (Figure 3J) and phosphatidylinositol 4-kinase (Figure 3K) showed a trend similar to that of the array data (Figure 2A), where GTPase RhoA showed fewer transcripts at the 4 or 12 h time points, and phosphatidylinositol 4-kinase showed lower mRNA expression at the 12 or 24 h time points in E2-treated samples compared to controls (Figure 3J and 3K). qRT-PCR also showed a significant increase in phosphatidylinositol 4-kinase transcript numbers at 24 hours of transition in controls compared to E2-treated samples (Figure 3K). We observed a significant drop in mRNA expression of GTPase RhoA by qRT-PCR in E2-treated samples compared to controls or to the baseline time point 0 h at a later time point (i.e., 216 h). This gene also showed low level of mRNA expression at the 4 h time point in response to E2 treatment (Figure 3J). Thus, these signaling genes likely have a critical role in dimorphic switch from M-to-Y.

Transporter and detoxification genes

The microarray showed that mRNA for several genes were highly expressed in response to E2 at the 12 h time point

| Table 2. Sequence analysis of E2 regulated gene and expression values in comparison to controls at various time-points during M-to-Y transition. |
|Gene product| Accession number (GI)| BLASTX (Fungi); E-value| M| E2-Control (Ratio)|
|---|---|---|---|---|
|6| Benzoate 4-monoxygenase cytochrome P450 (JM_33b9)| 226283340 1e-60| 1.49| 1.01 6.24 1.54 1.18 0.59 1.16|
|Major facilitator superfamily/benzoate transport domain (JM_101a4)| 226280741 4e-07| 3.53| 0.57 4.25 1.40 0.35 1.15 0.69|
|Iron/manganese superoxide dismutases (JM_135a9)| 22628384 2e-77| 0.78| 0.71 9.20 0.99 1.28 1.43 0.44|
|7| Ribosomal protein L33 (JM_39e12)| 226292079 3e-21| 0.71| 1.20 6.31 1.17 0.66 0.80 0.77|
|50S ribosomal protein L6 (JM_123f9)| 226282860 2e-94| 1.32| 0.72 5.79 12.5 2.06 0.59 0.97|
|Ribosomal protein P2 (JM_50b8)| 226278075 5e-17| 7.45| 1.37 11.5 0.39 7.60 1.06 1.25|
|8| WD40 repeat-containing protein (JM_78e7)| 226280913 6e-24| 1.52| 4.17 0.53 1.71 1.33 0.51 0.96|
|E3 ubiquitin-protein ligase ‘ubr11’ (JM_100g9)| 226282645 1e-08| 0.62| 1.64 4.56 0.85 1.38 1.28 1.02|
|Ubiquitin ‘ubi’ (JM_27)| 25128744 Control spot| 0.46| 0.68 0.09 1.4 10.0 1.41 0.58|
|9| Palmitoyltransferase ‘erf2’ (JM_104c12)| 226279299 0.0| 1.74| 0.07 0.46 0.66 0.28 2.63 0.97|
|Small GTPase RhoA (JM_74e7)| 226281788 1e-20| 3.42| 1.00 0.08 0.66 1.51 0.61 0.91|
|Phosphatidylinositol 4-kinase ‘LSB6 serine/threonine/tyrosine protein kinases’ (JM_109f6)| 226280727 1e-118| 0.98| 2.12 0.10 1.09 0.59 2.12 0.84|
|Protein kinases (serine/threonine) ‘sgk2’ (JM_13e12)| 226283417 3e-19| 0.02| 0.07 0.18 0.38 0.56 4.80 1.90|
|Arrestin domain-containing protein (JM_41c10)| 226285401 3e-73| 1.52| 1.85 5.28 0.31 0.33 1.75 0.63|
|10| K homology RNA-binding domain type I (JM_117f5)| 226285102 6e-74| 1.11| 0.96 4.29 0.79 5.49 1.07 0.73|
|RNA interference and gene silencing protein (JM_16e9)| 226285398 1e-53| 1.53| 0.67 4.28 1.35 0.58 1.25 1.06|
|11| Chromodomain-helicase-DNA- binding protein (JM_36f5)| 226280983 1e-157| 0.0006| 0.84 0.71 3.18 2.09 6.08 20.6|
|Acetyltransferase superfamily (JM_56g7)| 226285124 7e-06| 1.88| 0.75 5.63 1.94 1.95 0.40 0.18|
|Histone H2A (JM_83c7)| 226283178 4e-28| 1.66| 2.16 0.09 1.09 0.84 0.51 1.21|
|12| Membrane-associating Marvel domain (JM_85d6)| 226278699 1e-06| 0.0551| 2.36 0.02 6.48 0.64 0.79 0.05|
|PP loop ATPase superfamily protein (JM_10b8)| 226280392 2e-176| 0.0004| 1.14 0.43 1.57 0.89 15.2 20.1|

Geometric mean of linear values from microarray data: E2 (17β-estradiol) vs Controls (untreated control plus ethanol-treated control), M: Mycelia, 6: Oxidative stress, 7: Ribosomal proteins, 8: Ubiquitin-mediated protein degradation, 9: Signal transduction, 10: RNA processing, 11: Chromatin structure, 12: Others. GI: GenBank accession number from www.ncbi.nlm.nih.gov. BLASTX expect value ≤1e-04 was considered significant.
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These included benzoate 4-monoxygenase cytochrome P450, which may be involved with hydroxylation of E2 [44], major facilitator super family (MFS)/benzoate transport, and iron/manganese superoxide dismutases. In contrast, the stress responsive gene, cdr4, which encodes multidrug transporters of the ABC family, showed low mRNA expression in E2-treated samples at 4 h followed by a large increase in mRNA expression at 12 h compared to controls (Figure 2A). qRT-PCR data for cdr4 (Figure 3L) showed a trend similar to that of the microarray data. However, a significant drop in numbers of transcripts of cdr4 by qRT-PCR was observed in E2-treated samples at later time points (i.e., 144 and 216 h) in relation to the 0 h baseline.

Ubiquitin-mediated protein degradation

The gene encoding WD40 repeat-containing protein, E3 ubiquitin-protein ligase ‘ubr11’ and ubiquitin C-terminal hydrolase family proteins are involved in ubiquitin-mediated protein degradation and mRNA transcripts from these genes were highly expressed at 12 h in E2 treated samples compared to controls in the microarray (Figure 2A). qRT-PCR data for ubr11 showed a
Based on domain attribution of ORF2 in contig 1, a Pol of *P. marneffei* conserved hypothetical protein: ORF1, a retrotransposon gag alternata (GI: 6683624). Sequence domains were identified from each sample was normalized by using CT values obtained for the above the bars by one or more asterisks, which indicate the p value (* p shows the result of statistical comparison between C (controls) or E2 (E2-treated) samples at various time points and the 0 h baseline time point.

**Genes involved in energy metabolism**

Thirteen clones (mitochondrial or nuclear genes) related to energy production showed altered transcriptional profiles in response to the presence of E2 by microarray analysis (Figure 2A). NADH-dehydrogenase subunit II, cytochrome oxidase subunit II and ATP synthase F0 subunit-9, enzymes of complexes I, IV and V of oxidative phosphorylation, respectively, showed low mRNA expression at 4 or 12 h time points during M-to-Y transition in E2-treated samples, but at later time points (i.e., 72 h) showed higher mRNA expression, as did other subunits of complexes I and IV (Figure 2A). The gene encoding for isocitrate lyase, a key enzyme of the glyoxylate cycle, showed low mRNA expression at the 12 h time point in E2-treated samples, but later time points showed higher mRNA expression (Figure 2A). These results are suggestive that due to morphological block or delay in E2-treated samples, glyoxylate cycle or oxidative phosphorylation was under-utilized for ATP production during the initial stage (4 or 12 h) of M-to-Y transition. We also found the gene for α-ketoglutarate decarboxylase (Figure 2A) to have high mRNA expression at later time points (i.e., 72 h) in E2-treated samples during M-to-Y transition. Interestingly, the gene for triosephosphate isomerase showed high mRNA expression at the 12 h time point in E2-treated samples (Figure 2A). This isomerase enzyme interconverts dihydroxyacetone phosphate with glyceraldehyde-3-phosphate, which proceeds further into glycolysis.

**E2 regulates a retrotransposon element during M to Y transition**

After sequencing of differentially expressed elements on the microarray, we found 31 clones that had sequence similarity to a retrotransposon element. A lower level of mRNA expression was to E2 during M-to-Y transition. We considered the 25% of the genome [8], provide an overview of the response of and detoxification, cell signaling, and retro-transposable elements. Our microarray data, though it lacks a complete coverage of the genome (i.e., we estimate the microarray covers approximately 25% of the genome [8]), provide an overview of the response of *Paracoccidioides* to E2 during M-to-Y transition. We considered the microarray data as leads for further evaluation of transcripts by qPCR. These results corroborate those from previous studies on temperature-induced dimorphism, as well as extend the results on E2-induced inhibition of M-to-Y transition.

**Discussion**

We describe the effect of E2 on the transcriptional profile of *Paracoccidioides* during M-to-Y transition. E2 may block or delay the morphological transition by modulating gene expression of several functional categories of genes including: heat-shock response, cell wall maintenance/remodeling, energy metabolism, transporter and detoxification, cell signaling, and retro-transposable elements. Our microarray data, though it lacks a complete coverage of the genome (i.e., we estimate the microarray covers approximately 25% of the genome [8]), provide an overview of the response of *Paracoccidioides* to E2 during M-to-Y transition. We considered the microarray data as leads for further evaluation of transcripts by qPCR. These results corroborate those from previous studies on temperature-induced dimorphism, as well as extend the results on E2-induced inhibition of M-to-Y transition.
hsp70 and hsp90 as a co-chaperone protein [49,50]. The members of this family of DnaJ/hsp40 proteins are evolutionarily conserved and characterized by the presence of one or more J domains in their N-terminus; the J domain is important for the hsp40-hsp70 interaction and stimulation of ATPase activity [49,51]. In mammals, hsp90, hsp70 and hsp40 take part in the estrogen hormone-receptor hetero-complex to assist proper protein folding and activation by hormone binding [52]. In addition, in yeast, Ydj1/hsp40 mutants displayed high basal levels of steroid receptor activity in the absence of estrogen suggesting that Ydj1/hsp40 is important for the receptor regulation by the hsp90 folding pathway [53].

A critical morphological difference between mycelial and yeast is the composition of the cell wall. The primary cell wall constituents of both are glucans, chitin, proteins and lipids [6]. However, yeast have a larger percentage of chitin than do the mycelia [6]. Our data showed gfa1 and chitin synthase had lower mRNA expression in E2-treated samples at earlier time points.

In both M- and Y-forms, 40% of the cell wall is glucan. In the M-form, the wall consists mainly of β-(1,3)-glycosidic linkages, whereas the Y-form mainly contains α-(1,3)-glycosidic linkages; only 4–5% of the cell wall of the Y-form is β-glucan [38]. The significant drop in the mRNA expression of α- and β-glucan synthases, gfa1, Y20, hsp70, hsp90 and hsp40 at late time points in E2-treated samples supports the lack of morphological change into yeast. Remodeling of the cell wall and reorganization of membrane-lipid occurs as Paracoccidioides adapts to changes in temperature [56,57]. Our data reflect this for the control cultures and indicate that while M-to-Y transition is complete in control cultures, it has been inhibited in E2-treated cultures.

In addition to changes in structural components, the two morphological forms differ in energy metabolism. Yeast cells are able to utilize aerobic or anaerobic metabolism, including the glyoxylate pathway, for their energy needs, whereas mycelial cells

**Figure 4. Alignment of 19 retrotransposable sequences found in contig 1 that are differentially regulated in E2-treated samples in comparison to control.** Sequence domains were identified from conserved hypothetical protein (ORF1: Retrotransposon gag protein) of *Penicillium marneffei* (GI: 212525972), ORF2: Retrovirus polyprotein, putative, of *P. marneffei* (GI: 212525970) and hypothetical protein of *A. nidulans* (GI: 67524427).

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tend to be more aerobic [15,16]. Our data suggest that energy needs in E2-treated samples have been maintained through glycolysis.

We found evidence that *Paracoccidioides* responds to E2 by activating genes that might remove or metabolize the hormone. In other yeasts, E2 enters into the cells by diffusion [58]. Once E2 is activating genes that might remove or metabolize the hormone. In a mechanism for detoxification of E2, analogous to the efflux pump to remove the E2.

We also observed upregulation of transcripts of the major facilitator superfamily gene with a benzoate transport domain, benzoate 4-monooxygenase cytochrome P450 in E2-treated cultures; this protein shares significant amino sequence similarity with CYTP3A4 (E = 5e-13; GI: 6470135) and CYTP3A7 (E = 5e-23; GI: 50840237) in humans. Isoforms of the cytochrome P450A4 have strong activity in the formation of 2-hydroxyestradiol and 4-hydrox estradiol from E2 [44]. Induction of CYTP3A4 by E2 may be a mechanism for detoxification of E2, analogous to the hydroxylation of progesterone by *Trichophyton mentagrophytes*, which reduces the inhibitory effects of progesterone on growth [59]. Possibly, *Paracoccidioides* may reduce the intracellular burden of E2 by hydroxylation or by an efflux mechanism, to remove the inhibitory actions of E2 that prevent progression of dimorphism.

We found that many genes changing in mRNA expression involved signaling pathways. We observed down-regulation of mRNA of small GTPase RhoA (G-protein) and palmitoyltransferase ‘erf2’ in E2-treated samples. G-protein family proteins, such as ‘erf2’ is involved in the attachment of palmitic acid to G-proteins, and affects G-protein function by targeting it to a specific membrane domain, regulating responsiveness to upstream signaling [62]. Other genes associated with signaling, phosphatidylino- stol 4-kinase and protein kinase (serine/threonine), showed low mRNA expression in E2-treated samples. These genes are involved

### Table 4. Analysis of the sequenced clones showing sequence similarity with retrotransposon elements.

| Contigs/ singlets | Size (bp) | Pb01 (BLASTX) | Other fungi (BLASTX) (taxid: 4511) |
|-------------------|-----------|---------------|---------------------------------|
| Gl                | E-value   | Gl            | E-value                        |
| **Contig 1**      | 4511      | Hypothetical protein (Gl: 226283053) | 3e-46 | Pol A. alternate (Gl: 6683624) | 0.0 |
|                   |           | Hypothetical protein (Retropepsin) (Gl: 226284062) | 4e-45 | Hypothetical protein A. nidulans (Gl: 67538144) | 0.0 |
|                   |           | Hypothetical protein (Gl: 226277605) | 5e-35 | Retrotransposon gag protein P. marneffei (Gl: 212525972) | 3e-14 |
|                   |           | Hypothetical protein (Gl: 226286485) | 7e-31 | Retrovirus polyprotein P. marneffei (Gl: 212525970) | 0.0 |
|                   |           | Hypothetical protein (Gl: 226284993) | 7e-24 | Retrotransposon polyprotein T. stipitatus (Gl: 242760779) | 1e-95 |
|                   |           | Hypothetical protein (Gl: 226283163) | 2e-19 |                                           |     |
|                   |           | Hypothetical protein (Gl: 226279429) | 8e-15 |                                           |     |
|                   |           | Hypothetical protein (Gl: 226283120) | 3e-07 |                                           |     |
| **Contig 2**      | 1548      | Hypothetical protein (Gl: 226284593) | 1e-07 | Endonuclease/reverse transcriptase/Non-LTR retrotransposon T. stipitatus (Gl: 242825272) | 4e-93 |
|                   |           | Conserved hypothetical protein (Gl: 226283120) | 7e-09 | Hypothetical protein (Integrate core domain) A. nidulans (Gl: 67524427) | 2e-30 |
|                   |           | Conserved hypothetical protein (Gl: 226280246) | 8e-04 | Retrovirus polyprotein, (Integrate core domain) P. marneffei (Gl: 212530907) | 1e-28 |
| **Contig 4**      | 1335      | Hypothetical protein (Retropepsin) (Gl: 226277726) | 4e-100 | Retrovirus polyprotein T. stipitatus (Gl: 242775227) | 6e-14 |
|                   |           | Hypothetical protein (Reverse transcriptase/Ribonuclease H, Ty1/Copia family of RNase HI in long-term repeat retroelements) (Gl: 226285610) | 9e-27 | Hypothetical protein (Reverse transcriptase & RNase HI) A. capsulatus (Gl:154280503) | 4e-59 |
|                   |           | Hypothetical protein (RNase H) (Gl: 226280117) | 2e-12 |                                           |     |
|                   |           | Hypothetical protein (Integrate core domain) (Gl: 226284491) | 9e-06 |                                           |     |
| **Singlet 1**     | 1256      | No significant match | Reverse transcriptase P. chrysogenum (Gl: 255935443) | 1e-22 |
|                   | 859       | Hypothetical protein (Gl: 226285011) | 2e-05 | Retrovirus polyprotein (Reverse transcriptase) T. stipitatus (Gl:242810994) | 7e-04 |
|                   | 1168      | No significant match | Retrotransposon polyprotein (Reverse transcriptase) T. stipitatus (Gl:242805420) | 2e-17 |
|                   |           | Retrovirus polyprotein P. marneffei (Gl: 212545550) | 4e-16 |                                           |     |

Sequenced clones showing sequence similarities with retrotransposon elements are subjected to Sequencer software that resulted in 4 contigs and 4 singlets. Gl: GenBank accession number from www.ncbi.nlm.nih.gov. BLASTX expect value ≤1e-04 was considered significant.

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in the phosphatidylinositol signaling pathway, important for the remodeling and reorganization of cell wall and cell membrane [63].

Ca²⁺, calmodulin and calcineurin have been reported to be involved in morphogenesis and calcium homeostasis during M-to-Y transition in Paracoccidioides [64,65]. Bastos et al. [9] showed induction of Rho GTPase activating protein, UVSB phosphatidylinositol-3 kinase, myo-inositol-1-phosphate synthase and phosphatidylinositol transfer protein in a transcriptome analysis 22 h into M-to-Y transition by Pb01. Chen et al. [66] showed increased transcript numbers of adenylate cyclase, as well as a G-protein subunit (GPB1), with the onset of M-to-Y in P. brasiliensis. However, at a later time point (i.e., 240 h) decreased numbers of transcripts of GPB1 versus increased numbers of transcripts of G-protein subunits GPA1 and GPG1 were observed [66], indicating modulated expression of these genes control the signal needed for M-to-Y dimorphism. We observed reduced numbers of transcripts for small GTPase RhoA, palmitoyltransferase 'erf2', phosphatidylinositol 4-kinase and protein kinase, all involved in signal transduction, during initial stages of M-to-Y transition. This would reduce the magnitude of signaling during the dimorphic switch in E2-treated samples, and further implicates the critical role of signaling regulation in dimorphism.

In the early phase of transition in E2-treated samples, we observed increased mRNA expression for genes of E3 ubiquitin-protein ligase 'ubr11', WD40 repeat-containing protein, arrestin-domain-containing protein and ubiquitin C-terminal hydrolase. E3 ubiquitin-protein ligase is involved in the ubiquitination of the arrestin-protein complex formed with G-protein coupled receptor (GPCR), targeting the GPCR to the proteasome for proteolytic cleavage [67], effectively, terminating the signal or desensitizing GPCR signaling. It also plays a role in attenuation of cAMP signaling [67]. Others have shown elevated intracellular cAMP levels correlate with transcript expression of adenylate cyclase during M-to-Y transition by Paracoccidioides [66,68]. In Rhizopus nigricans, a plasma membrane-bound progesterone receptor coupled to G-proteins has been reported, where progesterone inhibits fungal growth by a G-protein-mediated decrease in intracellular cAMP [69].

The mRNA changes in these signaling pathways shown by our data may provide insight into the mechanism of E2 action. Although, the action of steroid molecules normally occurs through classic steroid receptors, it can also be mediated through alternative molecules residing in the membrane. The induced transcripts of E3 ubiquitin-protein ligase, arrestin-domain containing protein, WD40 repeat-containing protein and ubiquitin C-terminal hydrolase, and reduced transcripts of GTPase RhoA and palmitoyltransferase 'erf2', in E2-treated samples during initial phase of transition suggests that a WD40 repeat-containing protein may serve as an anchor for ubiquitination of the arrestin-protein by E3 ubiquitin-protein ligase when the arrestin is bound to GPCR or GPCR-like protein, directing them to the protein degradation pathway to terminate GPCR or GPCR-like protein mediated signaling in E2-samples. This suggests that E2 binds to a transmembrane protein that has affinity for G-proteins.

We identified several contigs or singlets, showing sequence similarity with retrotransposable elements. These elements were more highly expressed with the onset of M-to-Y transition in the controls than in the E2-treated samples. These data support the existence of retrotransposable elements in the genome of Pb01; transcripts of Ty-like elements have been reported in the Pb01 isolate [13]. Furthermore, we found a 198 bp long terminal repeat (LTR) similar to Ty-like elements, which include genes encoding both structural and enzymatic proteins, and remnants of retro-transposable elements. We showed mRNA expression of retrotransposable elements was modulated in the presence of E2 during M-to-Y transition. Although, expression of these elements may lead to deletion, insertion or translocation of genes in the genome, their role during M-to-Y transition requires further study. A genomic survey of P. brasiliensis strains revealed eight new Tc1/mariner families (i.e., DNA transposons), referred to as to TremA (Transposable element mariner) through TremH [70]. We found no Trem transposons in our sequence data.

Although evidence has been presented regarding how E2 inhibits morphological transition from mycelia to the parasitic yeast, questions remain. Confirmation that E2 binds to a true receptor in Paracoccidioides to modulate gene expression and the entire sequence of events that take place in the presence of E2 during M-to-Y inhibition remains to be elucidated. Nevertheless, our data provides insights into how exposure to exogenous E2 inhibits M-to-Y transition in Paracoccidioides, and how E2 effects could lead to the strong gender bias for the development of the clinical disease [4].

**Materials and Methods**

**Pb01 strain and maintenance**

We used Paracoccidioides lutzii strain Pb01 (ATCC MYA-826) to construct a random sheared gDNA array for evaluation of gene expression [8], similar to studies done in other laboratories [9,13,40]. M- and Y-forms were maintained on modified McVeigh-Morton medium (MVM) agar slants at ambient temperature and at 37°C respectively, and were transferred monthly [71].

**Pb01 growth and nucleic acid extraction**

M culture filtrate was filter sterilized from MVM broth cultures (Pb01) after 2-4 weeks of growth at 25°C on a gyratory shaker at 150 r.p.m. and stored at 4°C for use as a growth supplement. M spent culture medium was used as growth supplement (2:1 ratio of fresh liquid MVM to spent culture filtrate) as described previously for the growth of M liquid cultures [36,72]. Two-week-old M slants were used to inoculate 300 ml of supplemented liquid MVM media, and liquid cultures were grown for 8 d on a gyratory shaker at 150 r.p.m. at 25°C. After the 8 d growth period, 17β-estradiol (E2; Steraloids, Newport, RI, USA) was added to M-phase cultures at a final concentration of 2.6 x 10⁻⁷ M in 0.1% absolute ethanol or ethanol alone (final concentration 0.1%) [36]. This concentration of E2 has a high physiological concentration and was selected to achieve a high proportion of inhibition of M-to-Y transition. The physiological and pharmacological concentrations of E2 relevant to human females range from 2 x 10⁻¹⁰ M to 2 x 10⁻¹⁶ M [4].

Morphological transition from M-to-Y was induced by increasing the incubation temperature from 25°C to 37°C. Samples from 8 d old M cultures were collected, before adding E2 or ethanol alone and served as the 0 h time point. After the temperature switch, samples were harvested from untreated controls, ethanol-treated controls, or E2-treated cultures at 4, 12, 24, 72, 144, or 216 h time points by centrifugation at 1500 r.p.m. for 10 minutes followed by snap-freezing of the fungal mass in liquid nitrogen. These samples were stored at −80°C until RNA extraction was performed. Liquid cultures were evaluated for contaminating organisms by plating samples on 5% sheep blood agar (BD Diagnostic Systems, MD, USA) followed by incubation for 2–3 days at 37°C, and by microscopic examination of Lactophenol cotton blue (Becton, Dickinson and Company, Mexico) stained slides of Pb01; the slides were also used for morphological evaluation and confirmation of M-to-Y transition.
RNA extraction. Fungal cells were thawed in TRIzol reagent (Invitrogen, Carlsbad, CA) followed by cell disruption with 0.5 mm zirconia/silica beads (Biospec) using a BeadBeater apparatus (Biospec Products, Inc., Bartlesville, OK) at high speed setting (3 pulses of 12–15 sec). Total RNA was extracted using TRIzol reagent per the manufacturer's instructions. Contaminating gDNA was removed per the manufacturer’s instructions using an RNaseasy mini spin column (RNasey mini kit, Qiagen Science, MD, USA) and an RNase-free DNase set (50 (Qiagen, GmbH, Hilden)). The quantity and quality of the extracted RNA were assessed by $\frac{A_{260 \text{ nm}}}{A_{280 \text{ nm}}}$ ratio as well as by electrophoresis through a 1.5% agarose gel stained with ethidium bromide for the presence of intact 18S and 28S ribosomal RNA bands, visualized by UV transillumination.

DNA extraction. Genomic DNA (gDNA) was extracted from yeasts collected from 5-day-old liquid cultures (37°C, shaking at 150 r.p.m.) using DNeasy Plant Mini Kit (Qiagen, GmbH, Hilden) per the manufacturer's instruction. The quantity and quality of the extracted gDNA was assessed by $\frac{A_{260 \text{ nm}}}{A_{230 \text{ nm}}}$ ratio and electrophoresis through a 0.8% agarose gel stained with ethidium bromide and visualized by UV transillumination.

cDNA labeling, hybridization procedure and image acquisition

Total RNA (48 μg) was used for cDNA synthesis using anchored oligo-dT (5’ TTT TTT TTT TTT TTT TTV N 3’). Fluorescently labeled cDNA was made by incorporating Cy5 or Cy3 labeled dUTP during reverse transcription of polyadenylated (PolyA) mRNA. An equal amount of total RNA from untreated controls from each time-point was pooled to generate a Cy5-labeled cDNA reference sample. Samples from each time point were individually labeled with Cy3.

Cy3-labeled samples were competitively hybridized against the Cy5-labeled reference sample on a pre-hybridized microarray slide (random shear gDNA microarray [8]). cDNA labeling and pre-hybridization procedures were followed as previously described [8]. Samples of labeled cDNA for hybridization were concentrated using a Microcon YM-30 Centrifugal Filter Device (Millipore) and suspended to 40 μl with 3 x saline-sodium citrate buffer (SSC; Sigma-Aldrich, St. Louis, MO), 0.1% sodium dodecyl sulfate (SDS; Sigma-Aldrich). These samples were preheated at 100°C for 2 minutes, 37°C for 15–25 minutes, transferred onto a microarray slide, covered with a cover glass and placed in hybridization chambers. Hybridization was carried out at 50°C for 14 to 18 hours followed by washing, in order, with 2 x SSC with 0.03%SDS, 2 x SSC, 1 x SSC and 0.2 x SSC for 2–5 minutes in each solution. Slides were dried by centrifugation and scanned within 24 hours. Scanner settings used 10 μm pixel size and photomultiplier tube gains for both detection channels, 635 nm (Cy5-red color) and 532 nm (Cy3-green color) were 340 nm and 500 nm, respectively (GenePix® 4000B Microarray Scanner, Axon Instruments, Union City, CA). All hybridization experiments were done in duplicate using samples from each of two independent cultures.

Microarray data analysis

Microarray hybridizations were imaged using a GenePix scanner. Images were processed using GenePix Pro 6.1.0.4 software. GenePix files were imported into R/Bioconductor [73], using the limma package [74], removing spots having quality flags of ≤ 50 or less. Analysis of array backgrounds revealed evidence of spatial gradients, so background correction was applied to each array using the normexp method with an offset of 50 [75]. We applied the arrayQualityMetrics package [76]. We normalized between arrays using variance stabilization from the vsn package [77], allowing for 20 percent outliers. We then normalized within arrays using loess to remove non-linearity from the MA curves (M and A are defined as: $M = \log_2(I1) - \log_2(I2)$, $A = 1/2(\log_2(I1) + \log_2(I2))$, where I1 and I2 are the intensities of the two channels). Finally, we obtained standardized log2 ratios by dividing the log2 ratios of each array by its median absolute deviation. Initially, we tried to analyze the data using linear modeling in the limma package, but found that many of the top results were due to outliers in which only one of the replicate samples had an extremely high value. Therefore, we performed the analysis using a non-parametric rank product methodology [78], which is robust to outliers. For each time point we compared the E2-treated samples with pooled untreated control and ethanol-treated control samples, performing the rank product test for 1000 permutations. The controls were pooled to minimize or eliminate any effects that the vehicle for the E2, ethanol, might have on the analysis on a gene-by-gene basis. Genes that had a vehicle effect should show increased variability of expression in the pooled control samples and therefore lower the resulting t statistic. Pooling also increased the accuracy of our analysis by increasing the number of available control samples. We identified up- and down-regulated genes on the basis of estimated p-value with a cutoff of 1e-3 in the rank product test for all time points.

Sequencing and bioinformatics analysis

Differentially expressed genes were manually inspected to exclude outliers in the controls (pooled untreated control and ethanol-treated control). Ninety-two array elements with a distinguishing expression pattern between E2-treated samples and controls were selected for sequencing. For each clone, 5 μl of phage from the cloned stock was used as a template for PCR using Platinum® PCR SuperMix as per manufacturer’s instruction (Invitrogen) as described previously [8]. Amplified products were purified using QIAquick PCR purification kit as per manufacturer’s instruction (Qiagen, MD, USA). DNA sequencing reactions were performed from both forward and reverse primers using ABI Big Dye v3.1 sequencing chemistry at the Protein and Nucleic Acid facility at Beckman Centre, Stanford University. Each sequence was processed and aligned to generate contigs or singlets using the DNA analysis software SequencerSM v4.8 (Gene Codes Corporation, MI, USA). The BLASTX program [79] with a cutoff expected value of ≤1e-04 at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used as a match to assign gene and functions to the sequenced DNA clones.

Quantitative real-time PCR

Real-time PCR reactions were performed in a 384-well format using Applied Biosystem 7900HT Fast Real-Time PCR system (7900HT Fast System). PCR SYBER Green RNA-to-Ct 1-step kit (Applied Biosystem, Foster City, CA, USA) were used per the manufacturer’s instructions in all real-time PCR reactions. Each PCR reaction was performed in 20 μl total volume, with 100 ng of total RNA as a template. The thermo-cycling conditions were comprised of an initial step at 45°C for 2 min for reverse transcription, followed by 95°C for 10 min, and 40 cycles of 95°C (denature) for 15 s, 60°C (anneal/extension) for 1 min. A dissociation curve was performed for primer specificity. All reactions were done in triplicate. Negative control reactions included no template or no reverse transcriptase to check undesirable amplifications.

We used the β-tubulin gene from gDNA dilutions ranging from 5 to 3×10^7 genome copies in 10-fold increments to generate a
standard curve to determine copy numbers, with a single gene copy per genome [90,01]. A linear relationship was obtained by plotting the threshold cycle against the logarithm of known amount of initial template [92]. Accordingly, from our previous study and other gene expression studies on Pb01 [9,40], the β-tubulin gene was used as the normalizer. Primers were designed from an exon-exon boundary or with a gap of intron sequence or from the 3' exonic sequence; primer sequences are described in Table 3. Real-time PCR reactions were performed on RNA samples from two independent biological replicates, the same as used in the microarray hybridizations.

Statistical analysis

The significance of the difference of the mean values of the data in independent samples at given time-points in real-time PCR figures [e.g. at 4 h comparing control and E2; (4C vs 4E2)] was established by two-sample unpaired t-tests using GraphPad Software (GraphPad Prism v 5.0, GraphPad Software). To compare control or E2-treated samples, at all time points to the baseline time point (0 h) in real-time PCR data, one-way ANOVA with a Dunnett post-test was performed. Comparisons of the controls (i.e., untreated and ethanol treated control) in the microarray and qRT-PCR data were also carried out and showed significant differences in gene expression (p≤0.05) at single time points for few genes in the microarray data (e.g., membrane-associated marvel domain, major facilitator superfamily, and retropepsin), as well as for the qRT-PCR data set where β-1,3-glucan synthase, α-1,3-glucan synthase, and hsp90 showed a difference between the controls. However, comparison of individual controls with E2-treated showed the same trend, either up- or down-regulation of mRNA expression, as the comparison of expression in the presence of E2 with the overall expression in the pooled controls; data are presented based on the pooled controls. Although, the ethanol used as the vehicle control had some effect on several genes at a single time point, it had little or minimal effect on subsequent comparisons made when pooling the controls. Thus, we pooled the untreated and ethanol controls to increase the number of replicate control samples and to reduce false positive results due to the vehicle ethanol. To determine correlation between microarray and real-time PCR data, log10 transformed β-tubulin normalized relative expression value of a gene from controls or E2-treated samples from microarray was plotted against log10 value from real-time PCR at 0, 4, 12, 24, 72, 144 and 216 h time-points. Spearman and Pearson’s correlation coefficients were determined in GraphPad Software for the entire temporal timeframe. A p-value<0.05 was considered significant in all analysis.

Accession numbers

The GenBank accession numbers (dbGSS id) for DNA sequences are available under F1778766-F1779357 and JMI175074-JMI175450. The microarray data discussed in this publication have been deposited in MIAME compliant database - NCBI’s Gene Expression Omnibus [83] - and are accessible through GEO Series accession number GSE30466 [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30466].

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Author Contributions

Conceived and designed the experiments: JS JPM LFM KVC DAS. Performed the experiments: JS. Analyzed the data: JS TW JPM LFM KVC DAS. Contributed reagents/materials/analysis tools: DAS. Wrote the paper: JS TW JPM LFM KVC DAS.

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