Transcriptional analysis of Coccidioides immitis mycelia and spherules by RNA sequencing

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**Abstract.**

Coccidioides species are dimorphic organisms that transform from mycelia with internal arthroconidia in the soil to a tissue form known as a spherule. This process can be recapitulated in vitro by increasing the temperature, CO2 and other culture conditions. In this study we have quantified gene expression in mycelia, young and mature spherules. Highly up-regulated in young spherules, include a spherule surface protein and iron and copper membrane transporters. Genes that are unique to Coccidioides spp. are also over-represented in this group, suggesting that they may be important for spherule differentiation. Enriched GO terms in up-regulated genes also include oxidation/reduction, response to stress and membrane proteins. Down-regulated genes are enriched for transcription factors, especially helix-loop-helix andC2H2 type zinc finger domain-containing proteins which is consistent with the dramatic change in transcriptional profile. Almost all genes that are up-regulated in young spherules remain up-regulated in mature spherules, but a small number of genes are differentially expressed. Some mRNA from transposons was detected and most of these were up-regulated in spherules. Mature spherules express more HSP31, and amylase than young spherules and less tyrosinase. Although these results are similar to small number of previous RNAseq and microarray studies, a number of novel observations have been made.

**Keywords:**

**1. Introduction**

*Coccidioides immitis* and *posadasii* are primary pathogenic fungi that are primarily found in the desert regions of the Western United States, Mexico, Central and South America (Barker). It causes pulmonary infections that range from asymptomatic to severe and can disseminate beyond the lung. The organism grows as a mold in the soil and produces asexual spores, termed arthroconidia, within the mycelium. When the soil is disturbed the mycelium can be ruptured and the arthroconidia are released. If inhaled by a susceptible host, the arthroconidium differentiates into a form that is morphologically very different in the lung known as a spherule. In tissue the spherule enlarges and can form many reproductive endospores. Mature spherules rupture and release endospores, which can then differentiate into the next generation of spherules. This form, the spherule, is the disease-associated form of the organism. If the disease is self-limited the reproduction of spherules is limited but if the disease is severe spherules continue to proliferate and elicit inflammatory and immune responses. In addition to the role in human illness, spherules may play a role in the ecology of the organism by infecting desert rodents (Taylor, J).

Understanding the biology of the spherule is important because this is the parasitic phase of the organism that interact with the host. The immune response is made to the spherule, so the identification of spherule antigens is important for understanding the immune response. Furthermore, antifungal drug testing has been done almost exclusively with the mold form of the organism but drug targets specific to spherules may exist as well. For all these reasons, understanding the differences between transcriptional profiles of spherules and mycelia is important.

The morphological transition between mycelia and spherule forms of *Coccidioides* is dependent on sensing host environment, and this transition can be recapitulated in the laboratory by changing temperature and other growth conditions. *Coccidioides* species can grow in saprobic form at 22-30 °C, whereas culturing at 37- 42 °C and 5-20% CO2 in Converse media is required for arthroconidia to convert to spherules (Mead, Barker, 2020). Utilizing these conditions, whole genome-level transcriptional profiling studies of saprobic and parasitic forms have been performed (Whiston ; Johannesson). It has been found in both *C. immitis* and *C. posadasii* that about 1,300 genes are up-regulated in mycelia and about 1,900 genes are up-regulated in spherules and that expression of known virulence genes are upregulated in the spherules, linking morphology to the virulence traits. For example, the spherule outer wall glycoprotein, which is the outermost layer on the spherule, is expressed only in spherules (Cole). Another study compared mycelia to young spherules and mature spherules to mycelia and to each other using micro-array technology has been done and this study re-analyzes the RNA obtained in that study by strand-specific RNA sequencing (Viriyakosol).

In this study, we seek to understand the transcriptome differences between mycelial and spherule phases of *C. immitis*, as well as the early and mature spherules using RNA sequencing. Our results show that there are more than 1500 genes that are differentially regulated in spherules and mycelial phases of *C. immitis*. We have compared the up-and down-regulated genes to previous studies and, where possible, analyzed the function of differentially expressed genes.

**2. Methods**

2.1 Culture Conditions

C. immitis R.S. strain was grown as mycelia or spherules as previously described (Viriyaosol). Young spherules were cultured in Converse media for two days and mature spherules were cultured for eight days. During that time period very few spherules ruptured.

2.2 RNA extraction and purification

RNA extracted from mycelia and spherules in the previous microarray study (Viriyakosol) was used for analysis.

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2.3 Analysis of RNAseq data.

Duplicate sets of paired strand-specific reads were mapped to predicted mRNA sequences and quantified using Kallisto (Bray, N). Count table from the quantification were analyzed bby DESeq2 in R. The results were filtered for an adjusted p value < 0.05 and up-regulation and down-regulation for gene tables are defined as more than 2log2 spherules/mycelia FC (FC) or less than -2log2. This cut off is chosen to minimize the false positive identification of differentially regulated genes.

2.4 Functional analysis of genes

All up- and down-regulated genes were evaluated using data from FungiDB (<https://fungidb.org/fungidb/app>). Unique genes were identified by testing hypothetical proteins for Blastp matches to the Swiss-Prot database. C. immitis genes without a match with an e value < 10-8 to species other than Coccidioides spp. were considered unique.

GO analysis was done using FungiDB (<https://fungidb.org/fungidb/app>) and the FungiFun (<https://elbe.hki-jena.de/fungifun/>) websites. Go term enrichment was deemed signficant if the adjusted p value was < 0.05). Another approach to functional annotation was done by protein fold recognition using the PHYRE2 Protein Fold Recognition Server (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>).

3. Results

There was very little within group variance in the RNASeq data and almost all of it was explained by the phase of the samples (mycelia compared to young or mature spherule RNA).

The RNASeq gene expression from the three conditions tested were compared to each other and the results are shown in Figure 1and Supplemental Table 1. In both young and mature comparisons to mycelia 8% of genes were up-regulated and 16-18% were down-regulated. The number of differentially expressed genes comparing day 8 spherules to day 2 spherules was much smaller (1.4 – 2 %).

Fig. 1



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3.1 Young spherules

The 20 most up-regulated genes in day 2 spherules are shown in Table 1 (in a separate document). The most highly up-regulated gene is spherule outer wall glycoprotein (SOWgp). This protein is expressed in very large amounts on the external surface of spherules but not mycelia and is involved in pathogenicity (Cole, Yu). Two other spherule antigenic proteins (Expression-library immunization protein-1 and parasitic-phase-specific protein PSP-1) are also very highly up-regulated, as are three transporters, including a copper transporter. There are only three genes that are predicted to be copper transporters in Coccidioides immitis, so this is a substantial increase in transporter expression. Six of the twenty very highly expressed genes are only found in Coccidioides spp.

Genes that are unique to Coccidioides spp. are more common in the up- and down-regulated gene sets than in all genes. 21% of all C. immitis genes have no close homologs in other species and are therefore defined as unique. The differentially expressed genes have a higher fraction of unique genes (Table 2), which suggests that some genes unique to Coccidioides spp. may be important for spherule differentiation.

Table 2

Unique genes in differentially group

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Group | Total | Unique | % | p |
| All genesa | 5336 | 1105 | 21 | NA |
| Up-regulated | 400 | 147 | 37 | < 0.05 |
| Down-regulated | 849 | 300 | 35 | < 0.05 |

Legend A: Genes with adjusted p values < 0.05 in the DESeq2 analysis.

The median number of orthologs was lower in differentially expressed genes than in all genes, which is consistent with the observation that more differentially expressed genes were unique to Coccidioides spp. (Table 2). The median length of differentially expressed genes was also shorter than the median length of all genes.

Table 3

Orthologs and length in differentially expressed genes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Median | All genes | Up-regulated | Down-regulated | p |
| Number of orthologs/gene | 111 | 63 | 78 | < 0.05 |
| Lengtha | 579 | 285 | 233 | < 0.05 |

Legend A: Median length.

Enriched GO terms in the up-regulated genes included oxidation/reduction processes, integral membrane components, transmembrane transport and response to stress. One of the oxidation/reduction genes is superoxide dismutase. Several metal transporters, including two copper and iron transporters are also up-regulated, as was found in the most highly up-regulated genes. Cellular component GO terms associated with cell membranes are dramatically over-represented ( p < 10-4). In addition, 90/404 up-regulated genes have at least one predicted transmembrane domain, compared to 921/5403 total genes (p <0.05). However, there is no difference in the proportion of up-regulated genes compared to all genes with a predicted signal peptide.

Another approach to functional prediction is to compare the amino acid sequence to known three dimensional models (Kelly). This approach identified 300 functional annotations in the up-regulated young spherule genes, 108 of which have no PFAM description. Some of the up-regulated genes are predicted to code for dynein, toxins, transposon proteins and a peptidyl-prolyl cis/trans isomerase.

There are twice as many down-regulated genes in young spherules than are up-regulated, so many genes that are expressed well in mycelial are expressed poorly in spherules. Down-regulated genes were enriched for the cytochrome p450 superfamily, transcription factors and oxireductase GO terms. There are only five transcription factors up-regulated in day 2 spherules but 30 are down-regulated.

3.2 Comparison to previous studies in C. immitis

The RNA used in this study has previously been analyzed by microarray (Viriyakosol). The microarray study found that 246 genes (2.5% of the total) of were up-regulated more than 2log2 in young spherules. The number of differentially expressed genes in the current RNASeq analysis was significantly larger; 8% were up-regulated and 16% were down-regulated. However, 69% of the differentially expressed genes identified in the microarray study were also differentially expressed in this RNASeq study. Similar results were seen with the down-regulated genes. Microarray analysis identified 471 down-regulated genes compared to 850 identified by RNAseq; 69% of the genes identified by microarray were also down-regulated using RNAseq analysis. These results suggest that the RNASeq is the more sensitive technique for determining differential expression but differences observed in microarray tend to be found in RNASeq too.

GO enrichment analysis of the up- and down-regulated genes were similar in the two studies. Up-regulated genes in the microarray study were enriched for oxidation-reduction processes followed by terms for sulfate and sulfite biosynthesis. Enrichment of metal transporter and homeostasis genes was not seen. However, GO analysis of the down-regulated genes in the microarray study revealed enrichment of transcription factors. One finding of the microarray study was that 25 protein kinase genes were down-regulated in spherules. In the current RNNASeq study, 16 of these genes were found to be down-regulated.

Whiston has previously published a study comparing C. immitis spherules (day 4 maturity) to mycelia. Reanalyzing Whiston’s data using our pipeline found that 902 genes were differentially expressed by at least 1log2 spherule/mycelia FC in both studies. The FC values for differentially expressed genes in both studies are compared in Figure 2. There is a positive correlation between the two studies (73% of the genes are in the same quadrants) but there are also obvious exceptions. The difference in spherule maturity may account for some of these disparities.

Fig. 2



Legend: Genes with FC values > 1 or < -1 in the current study were selected. FC values of genes in the current study are shown on the X axis and the FC values of matched genes from Whiston’s study are shown on the Y axis. The regression line was derived by the linear model and the shaded area indicates the 95% confidence limits.

3.4 Mature spherules

Gene expression in mature spherules was also compared to gene expression in mycelia (Supplemental Table 1). There were many more genes up-regulated in mature spherules (960) than in day young (407), but most of the genes up-regulated in young spherules were also up-regulated in mature organisms. In contrast, there were many genes that were down-regulated in young spherules but not mature spherules (Fig. 3).

Fig. 3

Diagram

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Legend: Comparison of up- and down-regulated genes in young spherules and mature spherules. Blue is young spherules, yellow is mature spherules.

The enrichment of GO terms in the up-regulated genes were similar to the results in young spherules; oxidation/reduction, transmembrane transport and integral membrane component terms were highly enriched. In contrast, the most significantly enriched GO terms in the genes that were down-regulated in mature spherules were associated with microtube activity and kinesin.

Comparing these data to the microarray study, the number of differentially expressed day 8 spherule genes detected by microarray was much smaller than the results in this RNAseq analysis. However, as we saw in the comparison of day 2 spherules, in the genes found to be differentially expressed in the microarray experiment of day 8 spherules, were usually up-regulated (74%) or down-regulated genes (86%) in both types of experiments.

3.5 Mature spherules compared to young spherules

Gene expression in mature spherules was also compared to young spherules. A relatively small number of genes were differentially expressed. Genes that most differentially expressed are in Table 4. The most highly up-regulated gene is a homolog of HSP31, which is a methylglyoxalase, a chaperone stress-response gene in yeast. The expression of this gene increases in response to DNA replication stress (ref). A ferritin-like protein, involved in iron regulation and oxidation reactions is also up-regulated as are genes involved in mRNA splicing, transcription, and meiotic recombination. Expression of alpha-amylase-1 gene was also up-regulated; this gene is required for pathogenicity in Histoplasma capsulatum, but it’s role in the pathogenicity of Coccidioides spp. is unknown (Goldman). The most dramatically

down-regulated gene was tyrosinase, a gene that plays a central role in the synthesis of melanin. Two genes that influence beta-glucan metabolism were also down-regulated, as were two other influencing DNA replication.

In the microarray study 177 genes were up-regulated in the mature spherules vs young spherules comparison. Only 5 genes were found to be up-regulated in both studies. In the microarray study 113 genes were down-regulated compared to 58 in the RNASeq study and only 16 genes were down-regulated in both studies. It is not clear why the results from the two studies were so different.

3.6 Expression of transposable elements in young spherules compared to mycelia

There are 1,309 Gypsy, Copia and TcMar transposons in in C. immitis. We have previously found that proximity to a TE was associated with a lower level of protein-encoding gene expression in C. immitis mycelia (Stajich, Kirkland). In this study we have examined expression of TE mRNA in mycelia and young spherules. Only 350 TE had expression levels with an adjusted p < 0.05, suggesting that most TEs were poorly expressed. 230 were up-regulated in spherules and only 15 were down-regulated. Gypsy TE were the most common up-regulated transposons. Analysis of up-regulated predicted protein-encoding genes in young spherules has shown that a few of them are transposon proteins and a previous proteomic study found one transposon protein in C. posadasii spherules (Lake ). There were a total of 77 genes that were within 1 kB up- or down-stream of the up-regulated TE. The median FC of these genes was 1.95, so genes near the up-regulated TE were somewhat over-expressed in spherules.

4. Discussion

The transition from the environmental form to the spherule is required for pathogenic Coccidioides spp. The morphologic changes are dramatic – the organism changes from a mycelium with internal spores to a round structure that enlarges circumferentially and divides internally to form a large number of endospores (Sun, 1979). These are released and can differentiate into mature spherules. The importance of spherule maturation and spherule release is demonstrated by the observation that an engineered chitinase deletion mutant that does not form spherules is avirulent (Xue, Cole, 2009). For these reasons, understanding the transcription program of spherules as they mature is clearly important for comprehending the biology of this dimorphic fungus.

Our findings in this study confirm previous experiments showing that there are substantial differences in gene expression in spherules and mycelia ( , ). Transcriptome studies need to satisfy several basic goals: collecting accurate quantitative data about gene expression, using appropriate tools to analyze the data and compare gene expression in different biological states and using information about relative gene expression to make useful hypotheses about biological functions. The last goal is difficult to achieve.

GO analysis of genes found that were up-regulated in young spherules compared to mycelia showed that oxidation/reduction, membrane proteins and transport functions were highly enriched. Changes in expression of oxidation/reductions are to be expected since the atmosphere for mycelial culture is air (0.4% CO2) compared to 14% CO2 in spherule culture conditions. Some of the up-regulated transporters were metal ion transporters, including iron and copper transporters One copper transporter was up-regulated seven-fold suggesting that spherules may have a much higher need for metal ions, especially iron and copper, than hyphae do. Recent experiments in Paracoccidioides brasiliensis have reported that copper deprivation has a major effect on metabolism (Petito). In addition, a comparison of gene expression in C. posadasii spherules and an engineered chitinase deletion (Xue, Cole, 2009) that does not endosporulate found a major difference was up-regulation of iron and copper uptake genes, which is further evidence for the importance of iron and copper uptake for spherule and endospore formation (Mead, 2020).

A cluster of genes including an iron siderophore are induced by iron-deprivation and are required for pathogenicity in Histoplasma capsulatum (Hwang, L). Five of the six iron-related genes (including the siderophore) have homologs in C. immitis that are up-regulated in young spherules. These genes are tightly clustered ( < 25 kB) on contig 1 and have the up-stream regulatory sites for the GATA transcription factor Sre1 that have been identified in identified in H. capsulatum. The Blastomyces dermatitidis homolog of Sre1 has been knocked out and the resulting mutant is unable to differentiate from mold to yeast, emphasizing the importance of this transcription factor and iron acquisition in B. dermatitidis yeast formation (Gauthier, 2010). However, the C. immitis homolog of Sre1 (SreP) is down-regulated in young spherules. These results show that the organization of iron-related genes in C. immitis and H. capsulatum is very similar and that the C. immitis iron acquisition genes are up-regulated in spherules.

Both differentially expressed genes (both up-regulated and down-regulated in spherules compared to mycelia) were enriched for genes that were only found in Coccidioides spp. It is not clear whether these genes are important for differentiation to spherules, or are a consequence of spherule differentiation. The median length of differentially expressed unique genes is relatively short (147 amino acids) and 14% of them are predicted to be secreted proteins. The unique genes tend to be shorter than all the differentially expressed genes and much shorter than all genes. The function of these genes is unclear, since they have no close homologs.

Many genes that have previously been identified to be up-regulated in spherules have also been found to be up-regulated in this study. These include SOWgp (Cole, Yu), parasitic phase specific (Delgado N, Hung CY, Tarcha E, Gardner MJ, Cole), genes ureidoglycolate hydrolase, urease and allotoinase (Cole). One gene that has been found to be up-regulated in the yeast (or spherule) phase of all dimorphic fungi is 4-hydroxyphenylpyruvate dioxygenase (4-HPPD or HpdA) ( ). This gene is involved in tyrosine catabolism which plays a role in the synthesis of melanin (Boyce). Chemical inhibition of 4-HPPD blocks the formation of yeast in P. brasiliensis and deletion of the gene blocks differentiation to yeast in Talaromyces marneffei ( , ). There are two genes coding for 4-HPPD in C. immitis – one is up-regulated and the other is down-regulated. Boyce has described a cluster of genes involved in tyrosine catabolism in C. immitis and other pathogenic fungi. The expression of this cluster of genes is up-regulated when tyrosine is only nitrogen source ( ). C. immitis spherules are grown in media containing ammonium salts as the primary nitrogen salts and the expression of the genes in this cluster are down-regulated, as would be expected.

In Histoplasma capsulatum four transcription factors (ryp1-4) are required for differentiation into yeast (Sil). These transcription factors are needed for changes in the transcriptional responses to an increase in temperature that triggers yeast formation (Beyhan). These proteins Ryp-2 and ryp-4 (FacB) are up-regulated in spherules but the other two transcription factors are not.

The group of genes that were down-regulated in young spherules were enriched for transcription factors. The stu1 transcription factor was down-regulated 5-fold in spherules. This transcription factor has been found to be required for optimal hyphal growth in H. capsulatum (Longo, Rappleye), so the difference between expression of this gene in hyphal and the parasitic form is shared by these two pathogenic, dimorphic fungi. Eleven of 29 C2H2 type zinc finger domain-encoding proteins were also down-regulated more than 1log2 and only four were up-regulated (Table 5). The expression of HLH transcription factors was also down-regulated. Both of these transcription factors influences growth rate and differentiation in Neurospora crassa (Carrillo). Transcription factors with the fungal Zn(2)-Cys(6) binuclear cluster domain, the most common class of zinc finger protein in Coccidioides spp., are not nearly as differentially expressed.

The most revealing result from the analysis of mature spherules is that almost all the up-regulated genes in young spherules remain up-regulated. This suggests that this group of genes may be required for life in the spherule phase; examining the transcriptome of spherules of intermediate maturities would address this question. Analysis of spherules at earlier times in development than day 2 might shed light on genes that are involved in the initial steps in differentiation into spherules.

Comparison of young versus mature spherules found a small number of differentially expressed genes but several of them are interesting. A homolog of HSP31 is the most highly up-regulated suggesting that mature spherules may be responding to stress in a different way than young spherules. An FK506-binding protein is also up-regulated. This enzyme is of interest since the calcineurin inhibitor Cyclosporin A inhibits the growth of C. immitis in vitro and in vivo (Kirkland). Other have found that calcineurin plays a critical role in the growth and pathogenicity of other fungi (Steinbach, Perfect). The up-regulation of alpha-amylase AmyA is of interest because this protein is required for pathogenicity in Histoplasma capsulatum (Goldman). Tyrosinase, a gene that is crucial in melanin metabolism, is the most down-regulated.

We used culture in Converse media to obtain spherules but culture at 37- 42 °C and 5-20% CO2 in RPMI with 10% fetal calf serum is an alternative method and is more similar to mammalian conditions (Mead,Barker). Transcriptional analysis of spherules in those conditions would be interesting but that has not been reported.

The major conclusions of the analysis of the expression of predicted TE genes are that a relatively small fraction (27%) of them are expressed. 65% of those are up-regulated in spherules and 0.4% are down-regulated. A previous proteomic study has reported that gag proteins are expressed by C. posadasii (Lake). The significance of up-regulation of TE expression is unclear.

Although many of the differentially expressed genes were found in both this study and the previous microarray study there were some important differences. The published analysis of the microarray study did not include the preferential expression of unique genes or the differences in length that the current study does. The up-regulation of iron and copper transporters was not appreciated. The association of differentially expressed genes with the cell membrane was also not seen. The relative expression of TE was not evaluated in the microarray study. All in all, this re-appraisal of the RNA using RNAseq and more sophisticated analysis has been worthwhile.