**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).

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 30C, whereas culturing at 39C and 20% CO2 is required for arthroconidia to convert to spherules. Utilizing these conditions, whole genome-level transcriptional profiling studies of saprobic and parasitic forms have been performed (Whiston ; Johannesson). It has been shown that expression of known virulence genes are upregulated in the spherules, linking morphology to the virulence traits. Therefore, understanding the biology of the spherule is important because this is the parasitic phase of the organism. Understanding the immune response to the organism requires an understanding of genes that are preferentially, or exclusively expressed in the spherule. 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.

Previous transcriptional profiling studies have analyzed transcriptional profiles of spherules and mycelia (Whiston; Johannesson; Viriyasokol). Specifically, Whiston et al identified spherule- and mycelial-specific genes in two both *C. immitis* and *C. posadasii*, and found that about 1300 genes are upregulated in mycelia and about 1900 genes are upregulated in spherules (Whiston). They have shown that number of known virulence genes were upregulated in the spherules (Whiston). For example, the spherule outer wall glycoprotein, which is the outermost layer on the spherule, is expressed only in spherules (Cole). In addition, in a study by Johannesson et al, the differences between *C. posadasii* presegmented and endosporulating spherules were examined using microarrays. There were only 43 genes that were differentially regulated in two different spherule stages (Johannesson). [Talk about Viriyasokol results briefly]

In this study, we sought 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 211 many genes differentially regulated between day 2 spherules and mycelial phases of *C. immitis*. Moreover, there are1930 genes that are differentially regulated between early or late spherules. We have compared the up-and down-regulated genes to previous studies and, where possible, analyzed the function of differentially expressed genes.

**2. Methods**

Growing mycelia and spherules

AARON – I need this information from you.

RNA purification method

Strand specific sequencing

Kallisto mapping

DESeq

Up-regulated and down-regulated are defined as more than 2log2 spherules/mycelia FC (S/M FC) or less than -2log2(S/M FC).

* There needs to be section about genes that are only found by RNAseq (and not by microarray). I would predict that these genes would be small ones. Perhaps we can conclude that this study outperforms the previous microarray in number of ways, but the most important is that it may provide more info about small spherule-enriched transcripts which may be encoding virulence factors (toxins, etc.).

Abbreviations

Day 8/ Day2 FC – Day 8 spherule / day 2 spherule fold change

Up- or down-regulated- up- or down-regulated S/M FC greater than 2(log2) or -2(log2)

3.Results

3.1. Basic data

Jason and Jesus please check - We need more text – Is this right?

The RNASeq count data was transformed and modeled using the negative binomial distribution in DESeq2. About 50% of C. immitis genes were up- or down-regulated compared to mycelia with an adjusted p value less than 0.05 (See Supplemenntal Fig. 1).

There was very little within group variance in the RNASeq data and and almost all the variance was explained by the phase of the samples (mycelia compared to day 2 spherule RNA) (Fig 1 and Supplemental Fig 2).

Fig.1

PCA plot of day 2 spherule and myclia gene expression

Table

Description automatically generated

Legend M: Mycelia; S48: Day 2 spherules.

The RNASeq gene counts from the three conditions tested were compared to each other and the results are shown in Figure 1. In both day 2 and day 8 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. 2



The results of the DESeq2 S/M FC analyses are in Supplemental File 1 and annotation of the genes that up- and down-regulated in day 2 and day 8 spherules are in are in Supplemental File 2 (MAKE FILE Day2 Up and Down FungiDB).

3.2. Day 2 spherules compared to mycelia

Genes that are unique to Coccidioides spp. are more common in the up- and down-regulated gene sets than in all genes. 21% of C. immitis genes have no close homologs in other species and are defined as unique (defined as no Blastp matches to other species with an e vales less than 10-8). The genes that are differentially expressed have a higher fraction of unique genes. This association suggests that some genes unique to Coccidioides spp. may be important for spherule differentiation.

Table 1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Group | Total | Unique | % | p |
| All genesa | 5336 | 1105 | 21 | NA |
| Up-regulated | 497 | 154 | 38 | < 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 smaller than the median length of all genes.

Table 2

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 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.

NOTE – all terms have an adjusted p value less than 0.05

GO terms involving oxidation/reduction, transmembrane transport (especially copper and other metal ions), oxidation/reduction and abiotic stress are over-represented. Two of the three genes predicted to be copper transporters were up-regulated. Cellular component membrane GO terms 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 compared to all genes with a predicted signal peptide.

Analysis of the up-regulated genes using the FunCat ontology ( ) found that genes involved in carbohydrate metabolism and cellular transport were the most highly enriched. It is not clear why there is such a different set of functional predictions using these two ontologies, but cellular import is a common function.

Day 2 up-regulated genes



Another approach to functional prediction is to compare predicted three dimensional models to known structures (Kelly). This approach has identified 300 functional annotations in the up-regulated day 2 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 are identified in the up-regulated genes.

3.3 Down-regulated genes in day 2 spherules

There are twice as many down-regulated genes in day 2 spherules than are up-regulated, indicating that many genes that are expressed well in mycelial are expressed poorly in spherules.

Some of the enriched Molecular Function GO terms include heme binding, tetrapyrrole binding, oxidation/reduction and iron binding. All of these terms refer to oxidation/reduction enzymes and it is unsurprising that their expression would be down-regulated as mycelia differentiate into spherules in a high CO2 environment. GO terms for transcription factors are also enriched. There are only five transcription factors up-regulated in day 2 spherules but 30 are down-regulated. The cellular component GO terms associated with the cell wall are also over-expressed.

Fig. 4 Down-regulated day 2



Legend A: GO analysis of down-regulated genes in Day 2 spherules, all terms have an adjusted p value <0.05. B: FunCat analysis of down-regulated genes in Day 2 spherules, all terms have an adjusted p value <0.05.

Analysis of the down-regulated genes using the FunCat ontology found somewhat similar results. Terms associated with detoxification by cytochrome P450 were enriched as were those fungal cell types differentiation. However, the largest number of significantly enriched genes code for secondary metabolism.

3.4 Comparison to previous studies

3.4.1 Microarray study

The RNA used in this study was extracted from mycelia, day 2 spherules and day 8 spherule (Viriyakosol). This RNA has previously been analyszed by microarray (Viriyakosol). The microarray study found that 2.5% of genes were up-regulated in day 2 spherules and 4.8% were down-regulated. The number of differentially expressed genes in the current RNASeq analysis was 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 the RNASeq study.

As in the current RNASeq study 34% of the genes that are up-regulated by microarray in day 2 spherules are unique, but, in contrast, only 4% of the down-regulated genes are unique. The median length of the up-regulated genes in the microarray study was 250, which is similar to the value in the RNASeq study but the median length of down-regulated genes in the microarray study was 545, which is is not signifignicantly different than the value for all C. immitis genes.

GO analysis of the up-regulated genes in the microarray study showed that oxidation-reduction processes were the most enriched followed by terms for sulfate and sulfite biosynthesis. The most enriched FunCat terms were those associated with carbohydrate metabolism and homeostasis.

GO analysis of the down-regulated genes in the microarray study revealed enrichment of transcription factors and regulators of transcription, metal ion and heme binding and regulation of small GTPase signal transduction. The most enriched FunCat terms were different. They included fungal cell type differentiation, cell division, septum formation and hydrolosis and microtube terms.

3.4.2 Whiston (day 4 spherules).

Reanalyzing Whiston’s data using our pipeline found that 902 genes were differentially expressed by at least 1log2 S/M FC in both studies. The S/M FC values for differentially expressed genes in both studies are compared in Figure 5. There is a positive correlation between the two studies (73% of the genes are in the same quadrants) but there are also obvious exceptions.

Fig. 5



Legend: Genes with S/M FC values > 1 or < -1 in the current study were selected. The S/M FC values of genes in the current study are shown on the X axis and the S/M 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.

3.5 Day 8 spherules vs mycelia

3.5.1 Up-regulated genes

Gene expression in mature spherules (8 days after differetiation) was also compared to gene expression in mycelia. There were many more genes up-regulated in day 8 spherules (960) than in day 2 spherules (407), but most of the genes up-regulated in day 2 spherules were also up-regulated at day 8. Furthermore, the S/M FC values of the shared genes was also very similar (Fig. 6) .

Fig. 6



Legend Comparison of S/M FC values in Day 8 spherules and Day\_2 spherules. All selected genes had a S/M FC value of > 2 in Day 2 spherules.

The enrichment of GO terms in the up-regulated genes were similar to the results in 2 day spherules except that oxidation/reduction terms were even more highly enriched. The enrichment of FunCat terms was similar to GO terms in day 2 spherules. FunCat terms involving iron and copper acquisition, and homeostasis of metal ions were over-represented.

Fig 7

Up-regulated genes in day 8 spherules



3.5.2 Down-regulated genes

1014 genes were down-regulated (compared to mycelia) in day 8 spherules. The enriched GO terms in the down-regulated genes included those associated with microtubule movement, signal transduction, protein kinase activity, oxidation-reduction and transmembrane transport.

Fig. 8 Down-regulated day 8



3.5.3 Comparison to microarray study

219 genes were up-regulated and 334 down-regulated in the microarray study of day 8 spherules, so the number of differentially expressed genes was much smaller than the results in the RNASeq analysis (932 up-regulated and 998 down-regulated). 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 experiments.

Analysis of the GO terms for the up-regulated genes found that oxidation-reduction processes and integral membrane proteiens were enriched. The enriched FunCat terms included seconday metabolism, cellular import and transport. The primary GO term that was enriched in the down-regulated genes was associated with microtube processes.

3.5.1 Day 8 spherules compared to day 2 spherules

Gene expression in day 8 spherules was also compared to day 2 spherules. There was little differential expression of genes in the two spherules of different maturities. Some of the 80 genes expressed at higher levels in day 8 spherules than day 2 spherules were amylase, two protein kinases, peptidylprolyl isomerase and FK506-binding protein 1. Many of the up-regulated genes were hypothetical proteins.

Some genes in the 57 down-regulated genes were a kex protein, two kinesin family proteins, cell division cycle protein Cdc20, Beta-glucosidase, tyrosinase, NEK protein kinase, calmodulin-binding protein Sha1 and cyclin.

3.6.1 Comparison to microarray study

In the microarray study 177 genes were up-regulated in the Day 8 spherules vs Day 2 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. Only 16 genes were down-regulated in both studies. It is not clear why the results from the two studies were so different.

3.7 Expression of transposable elements in day 2 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 ( ). In this study we have examined expression of TE mRNA in mycelia and day 2 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 day 2 spherules has shown that a few of them are transposon proteins and a previous proteomic study found one transposon protein in C. posadasii spherules ( ). There were a total of 77 genes that were within 1 kB up- or down-stream of the up-regulated TE. The median S/M FC of these genes was 1.95, so genes near the up-regulated TE were somewhat over-expressed in spherules.

4. Discussion

Our findings in this study confirm previous experiments showing that there are significant differences of gene expression in spherules compared to mycelia ( , ). We chose to map the NGS reads to the predicted transcriptome, so only mRNA amounts were estimated. Coccidioides spp. genes have few introns and very few genes have recognized alternative transcripts, so an exon expression study is not a high priority. 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 very difficult to achieve.

Functional analysis of differentially expressed genes is problematic because the classification often depends on classification of homologs in other species. Furthermore, classification of genes is frequenly to broad to be useful. For example the oxidation/reduction term was enriched in both up- and down-regulated sets of genes. In addition, analysis using GO ontology and FunCat ontology frequently gave somewhat different results. Nevertheless, the classifications are useful tools in some instances. GO analysis of genes found that were up-regulated in day 2 spherules showed that oxidation/reduction, membrane proteins and transport functions were highly enriched. Some of the transporters were metal ion transporters, including iron and copper transporters One copper transporter was up-regulated seven-fold. Recent experiments in Paracoccidioides brasiliensis have reported that copper deprivation has a major effect on metabolism (Petito). This GO analysis suggests that spherules may have a much higher need for metal ions, especially iron and copper, than hyphae do. In addition, genes involved in carbohydrate metabolism were enriched, which seems reasonable given the extensive remodeling and enlargement of spherules. Up-regulated genes were found to be enriched both by GO enrichment and an independent prediction of transmembrane domains.

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 day 2 spherules. These genes are tightly clustered ( < 25 kB) on contig 1 and have the up-stream regulatory sites for Sre1 that have been identified in identified in H. capsulatum. The C. immitis homolog of Sre1 (SreP) is also somewhat up-regulated (1.69log2-fold) in day 2 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 day 2 spherules, perhaps because of sreP-induced transcription.

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 147 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. However, the short genes are unlikely to code for proteins with enzymatic activity (IS THIS TRUE?)

Some of the other up-regulated genes including those coding for the spherule outer wall, and PSP1 which are expressed exclusively in spherules ( , Delgado N, Hung CY, Tarcha E, Gardner MJ, Cole). Genes involved in purine degradation have been shown to be up-regulated in spherules and important for the pathogenicity of C. immitis (Cole, Wise). Three of the genes ureidoglycolate hydrolase, urease and allotoinase are up-regulated 1 - 2log2 fold in day 2 spherules. Peptidyl-prolyl cis/trans isomerase is also up-regulated more than 2log2 fold in day 2 spherules. 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). Other up-regulated genes include five short proteins that have homology to toxins. These may play a role in pathogenesis but none of them is predicted to have a signal peptide.

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.

One of the hsp70 genes is up-regulated in day 2 spherules. There are nine homologs of the gene coding for this chaperone in C. immitis RS. In Histoplasma capsulatum, expression of an hsp70 gene is up-regulated less than 24 h (Caruso, Medoff). Hsp30 is a stress response gene in Saccharomyces cereviseae (Fungal heat-shock proteins in human disease). There are two homologs of this gene in the C. immitis RS genome and one is up-regulated in day 2 spherules.

A previous study has identified a few genes that are up-regulated in the parasitic phase of several dimorphic primary pathogenic fungi, using data from microarray studies and RNAseq studies (Kirkland, A few shared up-regulated genes may influence conidia to yeast transformation in dimorphic fungal pathogens, doi: 10.1093/mmy/myw019.) This study has confirmed that most of these genes are up-regulated in the current analysis. These include a gene involved in glutamine metabolic process, a hypothetical protein, a glycerol dehydrogenase, one of two 4-HPPD genes , a MFS transporter; and a polyketide synthase.

A mutant C. posadasii strain in which chitinase 2 and chitinase genes were knocked out has been developed as an avirulent strain for vaccine development (Cole, Chung). The mutant strain does not complete endospore development, so spherules cannot reproduce in mammals. Mead and Barker have found that eight genes are expressed at least a 2-fold lower level in the mutant spherules than in wild-type spherules, suggesting that these genes may play a role in endosporulation (Mead, Barker). Five of these genes have orthologs in C. immitis and two of those, a protein kinase and a hypothetical protein are up-regulated. These genes are very interesting candidates for further study.

Although many of the differentially expressed genes were found in both this study and the 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 association of differentially expressed genes with the cell membrane was also not seen. The microarray study found that many protein kinase genes were down-regulated in spherules, but the current study did not confirm that finding. The relative expression of TE was not evaluated in the microarray study.

Comparing the results of our study to a previous study done by Whiston and co-workers shows

that the FC values correlate pretty well. Using 1log2 as a cut-off for up-regulation 76% of the genes were differentially regulated in both studies but with a 2log2 cut-off only 16% were up-regulated in both studies. Some of the reasons that might account for this difference include the difference in spherule maturity and the difference in sequencing techniques. The differences between the two studies emphasizes the need for multiple RNAseq studies in spherules of different maturity from different strains and species of Coccidioides spp. to draw strong conclusions about consistent differential expression in spherules and mycelia.

The genes that were down-regulated in day 2 spherules were enriched for transcription factors and oxidation/reduction terms. Thirty transcription factors are over-expressed in down-regulated group of genes, only five are over-expressed in the up-regulated group. 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 in hyphae and the parasitic form is shared by these two pathogenic, dimorphic fungi. The down-regulated group is also enriched for membrane proteins.

FunCat enrichment of down-regulated genes in day 2 spherules found that terms for cytochromeP450 and secondary metabolism were the most common. The glutathione-mediated detoxification, ubiquinone biosynthesis and tyrosine degradation pathways were enriched in this set of down-regulated (NOTE see hiddenPathwayEnrichmentResult\_day\_2\_down). It is difficult to integrate the decrease in expression into a clear hypothesis, but it is clear that a large number of oxidation/reduction, transcription factors and secondary metabolism genes are expressed at lower levels in spherules than mycelia.

Many more genes are up-regulated in day 8 spherules than in day 2 spherules, but almost all the genes that are up-regulated in day 2 spherules remain up-regulated at day 8. As would be expected, the function enrichment analysis shows a similar profile, except that oxidation/reduction functions are more enriched in day 8 spherules. The genes that are down-regulated in day 8 spherules include a number of kinesin genes and other genes that are annotated as microtubule cytoskeleton terms. Mycelia in some filamentous fungi transport chitin synthases to the hyphal tip in secretory vesicles that are powered by kinesin and myosin (Takeshita, N.). Since spherules are round that growth mechanism may not be as important. Genes involved in fungal cell type differentiation and budding, cell polarity and filament formation are also down-regulated, which is consistent with that notion.

Only a small number of genes are differentially expressed in day 8 compared to 2 spherules. This is surprising since the day 8 spherule is morphologically very different than day 2 spherules and has formed, but nnot released endospores. It is possible that the endospores within the day 8 spherules are have a similar expression profile to day 2 spherules. One should realize that spherules at time points between day 2 and day 8 were not studied, so changes in level of expression might be observed at those time points. More genes were differentially expressed in the day 8/day 2 comparison in the microarray. However, very few genes were classified as up- or down-regulated in both studies. The reasonnn for this major discrepam=ncy is unclear.

The major conclusion from the TE expression study is that some TE gene products are expressed in both spherules and mycelia and about 25% of them are expressed at higher levels in spherules than mycelia. We do not know the significance of this observation.

This is one study of a single isolate of C. immitis comparing mycelia to spherules at two time points. More studies with other isolates and species (C. posadasii) would make these results more generalizable since conclusions about differential expression can be drawn with more confidence if several studies are done. Furthermore, examining multiple time points would give us more information about the course of gene expression as mycelia differentiate into spherules and spherules mature. This study was done using spherules grown in a defined media (Converse media) incubated at 42° C in14% CO2 (Converse), which are conditions very different than in the host. Others have reported that can mycelia differentiate into spherules after three days in RPMI media in 10% CO2 at 39°C, which would be more similar to mammalian conditions (Galgiani, Barker), so investigation of gene expression in those circumstances would be of interest. The disadvantage of using these conditions is that there is much less published experience and comparisons of spherules grown in RPMI to spherules grown in Converse media would be needed. To best understand the interaction of spherules with the host immune response, determining the transcriptional profile of spherules in the host would be necessary.

Differential transcription studies only provide data about association of gene expression with a state of differentiation. They do not allow us to identify the genes required for differentiation to spherules, or pathogenesis in the host. Identifying those genes requires specific gene deletions or inhibition of transcription. Molecular Koch’s postulates can be satisfied if the gene can be re-introduced and the phenotype restored (). The data that we are reporting here are useful for forming hypotheses that may lead to those definitive experiments.

Furthermore, changes in the transcriptional profile are not the only changes associated with differentiation. Changes in chromatin structure, epigenetic profile, protein-DNA interactions, expression of microRNA and other types of transcription are frequently associated with differentiation. The fungal proteome and metabolome also undergo changes. Almost none of this data is available for Coccidioides spp., and translating data from related species is problematic. In addition, integrating all this information and developing a biologically informative model is a difficult challenge.

Overall summary

Despite the limitations of this study, we have found a number of differences in gene expression inn spherules and mycelia. Differentially expressed genes are frequently unique to Coccidioides spp. and shorter than average. Some of the most convincing functional enrichment in genes that are up-regulated in spherules include membrane transporters, especially iron and copper transporters and oxidation/reduction functions. Many genes were association with the cell membrane. Functions down-regulated in spherules include transcription factors, microtubules and intracellular motility. The data will hopefully provide hypotheses for evaluation of the role of genes in differentiating from mycelia to spherules.