Research

New signaling pathways govern the host response to *C. albicans* infection in various niches

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Candida albicans, the major invasive fungal pathogen of humans, can cause both debilitating mucosal infections and fatal invasive infections. Understanding the complex nature of the host–pathogen interaction in each of these contexts is essential to developing desperately needed therapies to treat fungal infections. RNA-seq enables a systems-level understanding of infection by facilitating comprehensive analysis of transcriptomes from multiple species (e.g., host and pathogen) simultaneously. We used RNA-seq to characterize the transcriptomes of both *C. albicans* and human endothelial cells or oral epithelial cells during in vitro infection. Network analysis of the differentially expressed genes identified the activation of several signaling pathways that have not previously been associated with the host response to fungal pathogens. Using an siRNA knockdown approach, we demonstrate that two of these pathways—platelet-derived growth factor BB (PDGF BB) and neural precursor-cell-expressed developmentally down-regulated protein 9 (NEDD9)—govern the host–pathogen interaction by regulating the uptake of *C. albicans* by host cells. Using RNA-seq analysis of a mouse model of hematogenously disseminated candidiasis (HDC) and episodes of vulvovaginal candidiasis (VVC) in humans, we found evidence that many of the same signaling pathways are activated during mucosal (VVC) and/or disseminated (HDC) infections in vivo. Our analyses have uncovered several signaling pathways at the interface between *C. albicans* and host cells in various contexts of infection, and suggest that PDGF BB and NEDD9 play important roles in this interaction. In addition, these data provide a valuable community resource for better understanding host-fungal pathogen interactions.

[Supplemental material is available for this article.]

The pathogenic fungus, *Candida albicans*, frequently grows asymptotically on mucosal surfaces as part of the normal microbiota. However, when the host becomes immunocompromised or when the bacterial microbiota are disrupted by the use of broad-spectrum antibiotics, *C. albicans* can cause mucosal diseases, such as oropharyngeal candidiasis (OPC) (Sangeorzan et al. 1994; Rhodus et al. 1997; Revankar et al. 1998; Redding et al. 1999; Willis et al. 1999) and vulvovaginal candidiasis (VVC) (Sobel 1985). *C. albicans* also has the ability to disseminate through the bloodstream and infect most organs in the body (Klepser 2006). Hematogenously disseminated candidiasis (HDC) (Rangel-Frausto et al. 1999; Bader et al. 2004; Hajjeh et al. 2004) has a 47% mortality rate despite administration of antifungals (Gudlaugsson et al. 2003).

In both the mucosal and disseminated forms of disease, *C. albicans* must adhere to and invade nonphagocytic host cells. For example, during the initiation of OPC, *C. albicans* invades oral epithelial cells. During HDC, this organism invades endothelial cells in order to escape from the vasculature and infect the deep

tissues. The observation that invasion-defective *C. albicans* mutants display attenuated virulence in murine models of OPC and HDC is evidence of the critical importance of invasion to virulence (Phan et al. 2000; Sanchez et al. 2004; Park et al. 2005; Chiang et al. 2007). Invasion into both cell types occurs in part by endocytosis, which is induced when the *C. albicans* invasins Als3p and Ssa1p interact with receptors expressed on the surface of endothelial and epithelial cells (Phan et al. 2007; Sun et al. 2010). These receptors include N- and E-cadherin, HSP90B1, the epidermal growth factor receptor (EGFR), and ERBB2 (also known as HER2) (Phan et al. 2005, 2007; Liu et al. 2011; Zhu et al. 2012).

As endothelial and epithelial cells are among the first host cells that interact with *C. albicans* during the course of infection, a comprehensive analysis of how these cells respond to the pathogen, and vice versa, is essential to understanding the pathogenesis of OPC and HDC. Previous studies have used microarrays to individually examine the transcriptional response of *C. albicans* to endothelial or epithelial cells (Müller et al. 2007; Barker et al. 2008; Lim et al. 2011; Ikuta et al. 2012; Moyes et al. 2014), as well as the transcriptional response of these host cells to infection by

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C. albicans (Sandovsky-Losica et al. 2006; Zakikhany et al. 2007; Park et al. 2009; Martin et al. 2011; Wachtler et al. 2011). Although these studies have contributed to our understanding of the interaction between pathogen and host, the full catalog of transcriptional changes is not complete owing to the limitations of microarrays, which include limited dynamic range and poor sensitivity to analyze low abundance transcripts.

RNA-seq (deep-sequencing of cDNA) is not subject to the limitations associated with microarray-based transcriptional assays. Gene expression profiling with RNA-seq is consistent with results obtained by microarrays (Liu et al. 2007; Marioni et al. 2008; Bradford et al. 2010; Malone and Oliver 2011) but is significantly more sensitive, with a much greater dynamic range (Wang et al. 2009). These traits and the probe-independent nature of RNA-seq make it possible to analyze the transcriptomes of multiple species (e.g., pathogen and host) simultaneously (Tierney et al. 2012; Westermann et al. 2012; Humphrys et al. 2013).

In this study, we analyzed the transcriptional profile both of *C. albicans* and host cells during in vitro infection of oral epithelial and vascular endothelial cells. Network analysis of the data set uncovered several signaling proteins that were not previously associated with the host response to any pathogenic fungus. We functionally validated the role of two of these pathways, platelet-derived growth factor BB (PDGF BB) and neural precursor cell expressed developmentally down-regulated protein 9 (NEDD9) using a siRNA approach. RNA-seq analysis of a murine model of HDC and vaginal samples collected longitudinally from women as they progressed from health to disease suggested that many of the same signaling pathways are activated during the course of mucosal and/or disseminated infections in vivo.

Results

RNA-seq of in vitro *C. albicans* infections

To understand the complex interaction that takes place between a fungal pathogen and host cells, we performed high-throughput sequencing of cDNA (RNA-seq) prepared from poly(A)-enriched RNA isolated from monolayers of primary human umbilical vein endothelial cells (HUVECs) and immortalized oral epithelial cells (the OKF6/TERT-2 cell line) (Dickson et al. 2000) that had been infected with *C. albicans*. The RNA preparations contained a mixture of mRNAs expressed by *C. albicans* as well as mRNAs expressed by the host cells. In order to reduce strain-specific responses, we infected each type of host cell individually with yeast-phase organisms of two different clinical isolates of *C. albicans* for which genome sequences are available, SC5314 and WO-1. After being added to the host cells, both isolates germinated and formed hyphae that progressively elongated (Fig. 1A). Exposure of *C. albicans* to the tissue culture medium also triggers hyphal morphogenesis as well as a corresponding transcriptional change (Bensen et al. 2004; Bruno et al. 2010). To ensure that the observed fungal gene expression changes were due to interaction with the host cell and not simply a response to the medium, we performed RNA-seq on time-matched controls in which each *C. albicans* isolate was grown in each type of tissue culture medium in the absence of host cells. Similarly, we performed RNA-seq on control, uninfected endothelial and epithelial cells. All sequencing reads were aligned to the human genome (Ensembl GRCh38) (Cunningham et al. 2015) as well as the reference genome sequences for each of the *C. albicans* strains (SC5314, CGD; WO-1, Broad Institute), and the samples were processed as depicted in Supplemental

Figure S1 and described in Methods. From all of the experiments combined, we obtained a total of 4×10^9 reads that mapped to the human genome (average 160.2×10^6 reads per sample) and 4.5×10^9 reads that mapped to the *C. albicans* reference genomes (averaging 105.3×10^6 reads per sample) (Supplemental Table S1). Our ability to obtain equivalent amounts of reads from the different organisms despite the considerable difference in genome size is a function of (1) the fact that we added five yeast cells to the infection for every human cell; and (2) the fact that *C. albicans* forms multinucleated hyphae over the course of the in vitro infection (Fig. 1A). Our ability to obtain approximately 100 million reads that mapped to each of the genomes in each of the mixed samples indicates that RNA-seq is a suitable approach to comprehensively analyze the transcriptome of both fungal pathogen and host in the context of an in vitro infection.

The transcriptional response of the fungi and host cells was determined after 1.5, 5, and 8 h. The 1.5-h time point corresponded to the initiation of host cell invasion (Fig. 1B), the 5-h time point corresponded to the induction of host cell gene expression, and the 8-h time point corresponded to fungal induction of host cell damage.

We defined differentially expressed genes as those with a minimum of twofold change in gene expression (P -value < 0.05) between *C. albicans* infected host cells compared to either uninfected host cells or *C. albicans* cells grown in the corresponding tissue culture medium in the absence of host cells. Despite the significant sequencing coverage that we obtained (between 91% and 95% of open reading frames in each sample had an RPKM > 0.1), we observed a minimal transcriptional response in *C. albicans*. Surprisingly, we found very few *C. albicans* genes (127 from SC5314; 181 from WO-1) that were differentially expressed in response to either of the host cell types at any of the time points. The number of genes differentially expressed during individual experiments ranged from 15 to 67 for strain SC5314 (Supplemental Table S2) and from four to 86 for strain WO-1 (Supplemental Table S3). Notably, only 11 genes were differentially expressed in both SC5314 and WO-1 in the same direction under the same conditions across both host cell types and all three time points (Supplemental Table S4). Taken together, these results suggest that *C. albicans* does not mount a robust core transcriptional response to the presence of these host cells and that many of the genes required for the interaction with host cells are expressed during growth in tissue culture media alone.

Analysis of host cell gene expression revealed 792 genes that were differentially expressed during at least one time point of the experiments. Three hundred eighty-eight genes were differentially expressed during the course of infection by endothelial cells, and 478 genes were differentially expressed during the course of infection by epithelial cells (Supplemental Table S5). The transcriptional responses of each of the human cell lines was relatively distinct, as evidenced by the small number of genes (up-regulated: nine, 27, and 15; down-regulated: one, one, and two, at 1.5, 5, and 8 h, respectively) that were differentially expressed in both of the cell lines at any given time point (Supplemental Fig. S2A,B). We were unable to identify human genes that were differentially spliced in response to the fungal infection in vitro model.

Upstream regulator analysis of host gene expression

We reasoned that gene expression changes in the host could be used to identify novel signal transduction pathways that are

Figure 1. Interaction of *C. albicans* strains with each of the host cell types. (A) Microscopic images of endothelial cells and epithelial cells infected with each *C. albicans* strain for 1.5, 5, or 8 h in vitro. (B) A quantification of endocytosis for each *C. albicans* strain in each of the host cell lines at 1.5, 5, and 8 h post-infection. Results are the mean $\pm$ SD of three experiments, each performed in triplicate.

modulated in response to *C. albicans* infection, and that these pathways might be relevant to pathogenesis. We used the Upstream Regulator Analytic from the Ingenuity Pathway Analysis software (Ingenuity Systems; <http://www.ingenuity.com>) to identify signaling proteins whose downstream signaling pathways were potentially activated or repressed during the course of infection. This software assesses the overlap between experimentally derived gene lists and an extensively curated database of target genes for each of several hundred known regulatory proteins. It then uses the statistical significance of the overlap and the direction of the differential gene expression to make predictions about activation or repression of these regulatory proteins.

Analysis of differentially expressed gene lists from each time point of each host cell line predicted the modulation of a total of 230 different signaling proteins with most of the activation taking place at the 5- and 8-h time points during endothelial cell infection and during the 1.5- and 5-h time points during epithelial cell infection (Fig. 2; Supplemental Table S6). Figure 2 depicts the signaling proteins with the highest predicted regulations. The utility of this approach was verified by our identification of multiple pathways that are already known to be activated by *C. albicans* infection. Known pathways predicted to be activated in one or both cell types included TNF (tumor necrosis factor), MAPK1/3 (mitogen-activated protein kinase 1/3), IL1A (interleukin 1 alpha), IL1B (interleukin 1 beta), ERBB2 (erb-b2 tyrosine kinase 2), and NFkB (nuclear factor kappa B), TLR7 (toll-like receptor 7), CD40 ligand, IL6 (interleukin 6), MAP2K1 (mitogen-activated protein kinase kinase 1), EGF (epidermal growth factor), MAPK8 (mitogen-activated protein kinase 8), IFNG (interferon gamma) and PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) (Orozco et al. 2000; Cannom et al. 2002; Villar et al. 2005; Moyes et al. 2010, 2014; Zhu et al.

2012;). Collectively, these results suggest that infection with *C. albicans* activates multiple signaling pathways, some cell-type-specific, in both endothelial cells and oral epithelial cells.

The analysis also predicted the regulation of several signaling pathways that have not previously been reported to play a role in the host response to infection by any fungal pathogen (Fig. 2; Supplemental Table S6). Specifically, upstream regulators that are predicted to be activated by infection included FOXL2 (forkhead box L2), NEDD9 (neural precursor cell expressed, developmentally down-regulated 9), PDGF BB (platelet-derived growth factor BB homodimer), PAF1 (RNA polymerase II associated factor), SELPLG (selectin P ligand), SYVN1 (synovial apoptosis inhibitor 1), IGF1 (insulin-like growth factor 1), NUPR1 (nuclear protein, transcriptional regulator 1), and PPRC1 (peroxisome proliferator-activated receptor gamma coactivator-related 1). Upstream regulators that are predicted to be repressed by infection included COL18A1 (collagen type XVIII alpha 1), NKX2-3 (NK2 homeobox 3), and S100A6 (S100 calcium binding protein A6). We chose two significantly up-regulated pathways, NEDD9 and PDGF BB, for functional follow-up. In endothelial cells, both the NEDD9 and PDGF BB pathways were predicted to be activated at both the 5- and 8-h time points. In epithelial cells, both pathways were predicted to be activated at all three time points (Figs. 2, 3A,B; Supplemental Table S6).

PDGFR and NEDD9 govern uptake of *C. albicans* into endothelial and epithelial cells

We next performed a series of experiments to test if the PDGF and NEDD9 pathways govern the interaction between *C. albicans* and host cells. Using qRT-PCR, we examined the expression of a subset of genes that are downstream from PDGF BB (PPP1R15A, SOCS3, DUSP1, and NR4A3) or NEDD9 (DDIT4 and PTGS2) following infection with *C. albicans* mutants that had homozygous deletions in *EFG1*, *ALS3*, and *OCH1*. Deletion of each of these genes confers decreased uptake into endothelial and/or epithelial cells (Park et al. 2005; Phan et al. 2007; S Filler and Q Phan, unpubl.). Consistent with our RNA-seq results, infection with wild-type *C. albicans* cells for 5 h induced the expression of each of these genes. Furthermore, infection with each of the mutants resulted in reduced activation of the PDGF BB- and NEDD9-target genes compared to both the matched wild-type and complemented strains (Fig. 3C). Our observation that invasion-defective mutants have decreased ability to induce PDGF- and NEDD9-target genes is consistent with a model in which activation of the PDGF BB and NEDD9 pathways is required for maximum invasion of *C. albicans*.

One mechanism by which *C. albicans* invades endothelial and epithelial cells is induced endocytosis (Filler et al. 1995; Phan et al. 2000; Park et al. 2005). We used a siRNA knockdown approach to determine if the PDGF BB and NEDD9 pathways are

Figure 2. Upstream regulator analysis of host gene expression. Each regulator was predicted by Ingenuity Pathway Analysis (IPA) to be activated (green, $Z\text{-score} > 2$) or repressed (red, $Z\text{-score} < -2$) during infection with *C. albicans* in either endothelial cells or epithelial cells during at least one time point. Depicted are the regulators with the highest predicted regulations ($Z\text{-score}$). Values represent $-\log_{10}(P\text{-value})$ for the overlap between the target gene set of each regulator and the list of differentially expressed genes. Black indicates no predicted activation or repression. Red circles indicate regulators that have not been previously associated with the host response to *C. albicans* infection.

required for the endocytosis of *C. albicans* by host cells. The PDGF BB signaling pathway is activated when PDGF BB binds to the PDGF receptor beta (PDGFRB) (Battagay et al. 1994). Knockdown of either *PDGFRB* or *NEDD9* resulted in a significant reduction in endocytosis of *C. albicans* by both endothelial cells and epithelial cells (Fig. 4A,B). We also attempted to knockdown both *PDGFRB* and *NEDD9* in the same cells. However, although knockdown of either *PDGFRB* or *NEDD9* alone caused no detectable cytotoxicity, combined knockdown of both proteins was synthetically lethal,

which precluded determination of the effects of this knockdown on fungal endocytosis.

To confirm our finding that signaling through PDGFRB was required for maximal host cell endocytosis of *C. albicans*, we analyzed the effects of two different small molecule PDGFR inhibitors on the endocytosis of *C. albicans*. Both AG1296 (Kovalenko et al. 1997) and PDGFR Tyrosine Kinase Inhibitor III (CAS 205254-94-0) (Matsuno et al. 2002) significantly reduced the endocytosis of *C. albicans* by endothelial cells and epithelial cells (Fig. 4C,D). Collectively, these results demonstrate the key role of PDGFRB signaling in the endocytosis of *C. albicans*.

E-cadherin and N-cadherin function as host cell receptors for *C. albicans* and are required for efficient endocytosis by host cells into epithelial and endothelial cells, respectively (Phan et al. 2005, 2007). We tested whether the decrease in endocytosis that we observe by knockdown of *PDGFRB* or *NEDD9* could be simply the result of reduced expression of the cadherins. Neither E- nor N-cadherin expression was affected by knockdown of *PDGFRB* or *NEDD9* (Supplemental Fig. S3). Collectively, our results suggest that *C. albicans* activates the *NEDD9* and PDGF BB signaling pathways in both endothelial and epithelial cells, which subsequently facilitates invasion into these cells in a manner that is independent of cadherin expression.

Several of the pathways, including PDGF BB and *NEDD9*, are also regulated in mice with hematogenously disseminated candidiasis

Since *C. albicans* must invade endothelial cells in order to escape from the vasculature and infect the deep tissues during the development of disseminated disease, in vitro infection of endothelial cells serves as a well-established model to understand the pathogenesis of HDC.

To test whether PDGF BB and *NEDD9* are activated during in vivo HDC infections, we performed RNA-seq analysis on kidneys of mice 24 h after they were inoculated intravenously with *C. albicans*. We obtained an average of 587.4×10^6 reads from each of four kidneys (two infected and two uninfected controls). Expression analysis revealed that 1024 genes were up-regulated in the infected kidney relative to uninfected control and 661 genes that were down-regulated (Supplemental Table S7). Among the newly identified *C. albicans* response pathways that we found to be regulated by *C. albicans*

Figure 3. Regulation of PDGF- and NEDD9-pathway targets during in vitro infection. (A,B) Differential expression of PDGF- and NEDD9-target genes during infection of endothelial cells (A) and epithelial cells (B). Values represent log fold change expression relative to uninfected controls (RNA-seq expression values during infection divided by expression during uninfected mock infection). Yellow indicates increased expression during infection; blue indicates reduced expression during infection. (C) Differential expression of PDGF- and NEDD9-target genes during infection with invasion-defective *C. albicans* mutants. Values represent fold change differences in induction by mutant strain divided by induction by appropriate wild-type strain as determined by qRT-PCR.

infection in vitro, our upstream regulator analysis predicted the regulation of several of these during the in vivo kidney infection (Supplemental Table S8), including FOXL2, PAF1, SELPLG, SYVN1, NUPR1, COL18A1, and S100A6. Notably, our analysis also predicted the activation of both the PDGF BB (P -value of overlap = 1.62×10^{-26}) and NEDD9 (P -value of overlap = 1.09×10^{-7}) pathways (Supplemental Table S8). Figure 5 depicts the increase in gene expression of PDGF BB- and NEDD9-target genes in infected kidneys relative to uninfected kidneys. These results indicate that *C. albicans* infection activates both the PDGF BB and NEDD9 pathways in mice with disseminated candidiasis.

Transcriptomic evidence for activation of PDGF BB and ERBB2 pathways during vaginal candidiasis in humans

We next investigated whether the PDGF BB and NEDD9 pathways would be activated during *C. albicans* infection in humans. The best way to perform such an experiment would be to assay the same tissue from the same individual both prior to and during an infection. To this end, we took advantage of a preexisting clinical study focused on understanding the dynamics of the vaginal microbiome. In this study, vaginal samples were collected daily from 135 nonpregnant women over the course of 10 wk (Ravel et al. 2013). To test if the PDGF BB and NEDD9 pathways are activated during clinical episodes of vaginal candidiasis, we performed RNA-seq using RNA isolated from vaginal swabs that were collected as part of this study. Our analysis included samples from two women who were clinically diagnosed with an episode of vaginal candidiasis at least once during the course of the study (Ravel et al. 2013). In each case, the first sample (healthy) was collected when the subject was experiencing no vaginal symptoms and the second sample was collected when the subject was experiencing a clinically diagnosed episode of vaginal candidiasis (Fig. 6). An average of 18×10^6 reads per sample were obtained (Supplemental Table S9). In a focused analysis of these data, we found that 26 known PDGF BB-target genes were up-regulated during infection in Subject 12, and 17 of PDGF BB-target genes were up-regulated in Subject 40 (Fig. 6). We also found up-regulation of many target genes from two of the novel upstream regulators (SYVN1 and SELPLG) (Supplemental Fig. S4) that were predicted to be activated in both our in vitro infections (Fig. 2) and our in vivo mouse infections (Supplemental Table S8). A similar analysis did not indicate that the NEDD9 pathway was activated during vaginal candidiasis. These results suggest that the PDGF BB, SYVN1, and SELPLG

Figure 4. PDGFRB and NEDD9 mediate endocytosis of *C. albicans* by host cells. Effects of siRNA knockdown of PDGFRB or NEDD9 on endocytosis of *C. albicans* by endothelial cells (A) and oral epithelial cells (B). The effect of pharmacological inhibition of the PDGF receptor on endocytosis by endothelial cells (C) and oral epithelial cells (D) was also tested. Results are the mean $\pm$ SD of three experiments, each performed in triplicate. (*) $P < 0.00003$ versus controls; $n = 12$ from four independent experiments.

Figure 5. PDGF BB and NEDD9 are activated during disseminated candidiasis. Differential expression of PDGF- and NEDD9- target genes, as indicated by IPA analysis, in two uninfected mouse kidneys (mock) and two *C. albicans*-infected mouse kidneys 24 h after injection (24 h). Plotted are log transformed RPKM values that have been normalized across all four samples. Yellow indicates high gene expression; blue indicates low expression.

pathways are activated during vaginal candidiasis in humans, whereas the NEDD9 pathway may only be activated during disseminated infections.

Previous work has demonstrated that the host receptor kinase ERBB2 is autophosphorylated in oral epithelial cells during interaction with *C. albicans*, and this receptor plays a key role in the interactions of *C. albicans* with oral epithelial cells, both in vitro and in the mouse model of oropharyngeal candidiasis (Zhu et al. 2012). This pathway was also predicted to be activated in our in vitro infections (Fig. 2) and in vivo mouse infections (Supplemental Table S8). To search for evidence that the ERBB2 pathway is activated in vaginal candidiasis, we searched for known ERBB2-target genes

that might be differentially expressed in our longitudinal samples. We observed that 36 and 19 known ERBB2-target genes in Subjects 12 and 40, respectively, had increased expression during episodes of vaginal candidiasis. Therefore, these data indicate that the ERBB2 pathway is also activated during vaginal candidiasis in humans.

Discussion

In this study, we performed the most comprehensive transcriptomic analysis of host-pathogen interaction, for any bacterial or eukaryotic pathogen, to date. By performing RNA-seq on mixtures of RNA isolated from human endothelial and epithelial cells infected with *C. albicans*, we generated a data set of 8.5 billion mapped reads (4 billion for human and 4.5 billion for *C. albicans*) that will serve as an excellent genomic resource for the study of host-pathogen interaction in general. We used network analysis of our data set to identify host signaling pathways that are activated by *C. albicans* infection and identified ones that have not been previously tied to fungal infection. Our in-depth analysis of two of these novel pathways provides evidence that PDGF BB is activated during both disseminated and mucosal infection in vivo, whereas the NEDD9 pathway is activated during HDC.

Figure 6. PDGF-target genes are induced during clinical episodes of vaginal candidiasis. (A) Schematic representing the symptomatology and treatment of both subjects over the course of 5 wk. The arrows indicate the samples that were subject to RNA-seq. (NS) No symptoms were observed during sample collection; (VC) the sample was collected during an episode of vaginal candidiasis. (B) Heatmaps depicting the expression (normalized RPKMs) of PDGF BB-target genes with a minimum 1.5-fold change between the healthy samples (NS) and diseased (VC).

To our knowledge, only two other studies, both microarray-based, examined the response of epithelial cells or endothelial cells to *C. albicans* using a similar time-matched negative control sample that consisted of fungal cells incubated in tissue culture media without host cells (Park et al. 2009; Wachtler et al. 2011). The list of genes that were found to be differentially expressed does not overlap significantly with either of these previous studies. For example, of the 127 genes that were differentially expressed during at least one time point in one cell type in the current study, five were reported by Park et al. (2009) and 11 were reported by Wachtler et al. (2011) to have changed in the same direction. There are several possible explanations for the poor concordance. For the Wachtler study, the analysis was done with a different cell line (polarized TR146 oral epithelial cells) at 20, 60, and 180 min post-infection (Wachtler et al. 2011). The authors observed the most robust transcriptional response at 20 min post-infection with the majority of the gene expression changes disappearing by 60 min post-infection. Therefore, the most likely explanation for the discordance with this study is the difference in host cells and the timing of the experiment. For the Park study, the analysis was done on FaDu oral epithelial cells at 45, 90, and 180 min post-infection with clinical isolates that differ from the *C. albicans* strain, SC5314 (Park et al. 2009). So, the most likely explanation for the discordance with this study is the combination of different host cells as well as the different *C. albicans* strains examined.

Because of the large number of host genes whose transcript levels changed in response to *C. albicans* infection, we focused our analysis on upstream regulators, which we define as any protein or protein complex that is known to govern the mRNA abundance of another gene. These molecules play important roles in governing many cellular pathways including those involved in the host response to microbial infection. Their potential to regulate the expression of functionally related or redundant genes makes them a valuable starting point for functional follow-up studies of large transcriptomic data sets. To identify upstream regulators in the host that might be activated or repressed in response to infection by *C. albicans*, we performed network analysis of the gene expression data using the Upstream Regulator Analytic from the Ingenuity Pathway Analysis software. This approach was validated by the prediction of many signaling proteins that have already been demonstrated to respond to infection by *C. albicans*.

We observed a significant overlap in predicted pathway activation between the two cell lines at the later time points (Fig. 2) despite a very small overlap in coregulated genes at the same time points (Supplemental Fig. S2). In order to get a positive hit in our analysis, we need a statistically significant overlap between our data set and the list of target genes for a given pathway curated in the IPA database. The high level of overlap in the predicted pathways is the result of the endothelial cells and epithelial cells each expressing a different subset of genes in each of the pathways, and the list of differentially expressed target genes in each cell line is large enough to allow for each of the two minimally or non-overlapping sets to still be statistically significant.

We focused our functional follow-up on the two novel pathways that were significantly activated during our in vitro infection experiments: PDGF BB and NEDD9. PDGFs are serum proteins that stimulate cellular migration and have well-established roles in embryonic development and human diseases such as cancer and atherosclerosis (Kohler and Lipton 1974; Ross et al. 1974; Westermarck and Wasteson 1976; Demoulin and Essaghir 2014). The PDGFs are encoded by four different genes—*PDGFA*, *PDGFB*, *PDGFC*, and

PDGFD—and function as secreted homodimeric proteins with distinct biological roles (Demoulin and Essaghir 2014). We found significant overlap between the set of genes that are induced in response to *C. albicans* infection and the set of genes that are regulated by the interaction of the PDGF BB homodimer with its receptor. This significant overlap, along with the directionality of the gene expression changes, is consistent with activation of cellular signaling via the *PDGFRB* subunit. We determined that siRNA knockdown of the *PDGFRB* subunit in both endothelial and epithelial cells significantly reduced the capacity of each cell type to endocytose *C. albicans* cells. In addition, invasion-defective *C. albicans* mutants were less effective at inducing the expression of PDGF BB dependent genes, as assayed by qRT-PCR. These results suggest that *C. albicans* uses PDGF signaling as a mechanism to invade barrier cells by induced endocytosis. This mechanism of invasion has not been described for a fungal pathogen; however, one study suggests that *PDGFRB* promotes cellular uptake of the intracellular pathogen *Chlamydia trachomatis* (Elwell et al. 2008).

Although we observed significant reduction of the endocytosis upon knockdown of *PDGFRB*, host cell invasion was not completely abrogated. This can be explained by the fact that *C. albicans* is known to interact with other cellular receptors to induce its own endocytosis including *ERBB2*, *EGFR* (Zhu et al. 2012), and *N-cadherin* and *E-cadherin* (Phan et al. 2007). Furthermore, although *PDGFRB* is the principle receptor for PDGF BB, this molecule can also bind to the *PDGFRA* subunit homodimer or *PDGFR A/B* heterodimer. Therefore, the possibility exists that *C. albicans* can also signal through the alpha subunit (Demoulin and Essaghir 2014). The *PDGFR* inhibitors that we used in our experiments inhibit both *PDGFRA* and *PDGFRB*, which may explain why they caused a greater reduction in host cell endocytosis of *C. albicans* than siRNA knockdown of *PDGFRB*.

NEDD9 is a member of the Cas family of scaffolding proteins upon which many signaling pathways converge, and it is implicated in many aspects of cancer biology (Cabodi et al. 2010). Although *NEDD9* is known previously to be expressed by epithelial cells and T cells, we found that it is also expressed by endothelial cells. Several lines of evidence point toward a scenario in which *NEDD9* transduces *C. albicans*-stimulated signals to promote induced endocytosis in both endothelial and epithelial cells. First, there was significant overlap between the *C. albicans*-induced genes and known *NEDD9*-target genes; and the directionality of these expression changes are consistent with activation of *NEDD9* by *C. albicans* infection. Second, siRNA knockdown of *NEDD9* resulted in reduced uptake of *C. albicans* by both endothelial and epithelial cells. Third, invasion-defective *C. albicans* mutants simulated the expression of *NEDD9*-target genes only weakly. *NEDD9* has been shown to be essential for transduction of signals that result from stimulation by PDGF (Natarajan et al. 2006) as well as overexpression of *ERBB2* (Little et al. 2014). Our data suggest that *NEDD9* may govern the host cell response to *C. albicans* by transducing signals from both the PDGF BB and *ERBB2* pathways. On the other hand, our finding that dual knockdown of *NEDD9* and *PDGFRB* was synthetically lethal indicates that these two proteins also function in parallel pathways that together are required for cell survival.

The results of our in vitro experiments suggest that PDGF BB and *NEDD9* play a role in the host response to *C. albicans* in vascular endothelial cells, which are relevant to HDC, and oral epithelial cells, which are relevant to mucosal infections. To look for evidence that these pathways are activated during in vivo infection, we characterized the host response to *C. albicans* infection using

a murine model of HDC and in women with vaginal candidiasis using RNA-seq. We found significant transcriptomic evidence of PDGF BB activation in both the murine HDC model and during the clinical episodes of vaginal candidiasis. These results indicate that *C. albicans* activates PDGFRB, either directly or indirectly during both disseminated and mucosal candidiasis. On the other hand, our in vivo transcriptomic analyses suggested that *C. albicans* activates NEDD9 during HDC but not during vaginal candidiasis. However, the lack of detection could be due to the relatively low coverage of the human genome that we were able to achieve in the vaginal samples. The low coverage is most likely the result of a significant number of sequencing reads originating from the bacterial microbiota. Whether disseminated infection with other microbial pathogens also activates the NEDD9 pathway is currently unknown.

A previous study has raised the question as to whether the response of mice to a pathogen has any relationship to the response of humans (Seok et al. 2013). However, the work presented here shows that the human response to VVC has significant similarity to both the mouse response to HDC and the in vitro response of epithelial and endothelial cells to *C. albicans* infection. Therefore, our results demonstrate the power of RNA-seq and network analysis, across multiple infection models, including murine models, to identify novel pathways that govern the interaction between fungal pathogens and the human host during invasive and mucosal infections. To our knowledge, this is the first study to analyze the host response to microbial infection in longitudinally collected clinical vaginal samples. Specifically, these results suggest that at least PDGF BB and NEDD9 have important roles in *Candida*–host interactions. Additionally, our significant data set will serve as a valuable community resource to better understand host–fungal pathogen interactions as well as to improve the human genome annotation.

Methods

Fungal strains and host cells

Both of the wild-type *C. albicans* strains used in this study (SC5314 and WO-1) were derived from clinical infections and are therefore both human derived. Strain SC5314 was originally recovered from a patient with generalized candidiasis at the Columbia College of Physicians and Surgeons, New York City (Odds et al. 2004). Strain WO-1 was isolated from the blood and lungs of a patient suffering from systemic candidiasis at University Hospitals, Iowa City, Iowa, in 1984 (Slutsky et al. 1987). The genetically engineered *C. albicans* strains used in this study are listed in Supplemental Table S10. Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cord veins and cultured as previously described (Filler et al. 1995). The human oral epithelial cell line, OKF6/TERT-2, was kindly provided by J. Rheinwald (Harvard University, Cambridge, Massachusetts) (Dickson et al. 2000) and was cultured as previously described (Phan et al. 2007).

Isolation of RNA from epithelial and endothelial cells

HUVEC and OKF6/TERT-2 cells were grown in a 10 × 15-mm tissue culture dish. For HUVECs, the medium was M199 (Life Technologies) containing 10% fetal calf serum and 10% fetal bovine serum. For OKF6/TERT-2 cells, the medium was DMEM (Life Technologies) without serum. The host cells incubated 5% CO₂ at 37°C until confluent, at which time 3 × 10⁷ *C. albicans* yeast were added to each dish for a multiplicity of infection of five yeast cells for every one human cell. After 90 min, 5 h, and 8 h of infection,

the cells were scraped from the plate and the human and fungal RNA was isolated using the Ribopure Yeast Kit (Ambion), according to the manufacturer's instructions. As a negative control, 3 × 10⁷ *C. albicans* yeast were added to the tissue culture plates containing medium alone without host cells and processed in parallel.

Murine model of disseminated candidiasis

Male BALB/c mice were infected via the lateral tail vein with 10⁶ yeast cells of *C. albicans* SC5314. After 24 h of infection, two infected mice and two uninfected mice were killed. Their kidneys were harvested, snap frozen in liquid nitrogen, and stored at –80°C. To isolate the RNA, the kidneys were homogenized and then subjected to bead beating with zirconium beads. Next, total RNA was extracted using the RNeasy mini kit (Qiagen; Cat #74104) using the washing and elution steps in the Qiagen Quick-Start Protocol. The animal experiments were approved by the Animal Care and Use Committee at the Los Angeles Biomedical Research Institute.

Processing of vaginal swabs

After obtaining informed consent, vaginal swabs were prospectively collected daily for 10 wk from 135 women (Ravel et al. 2013). Two samples were from each of two women who developed vaginal candidiasis prior to the development of symptoms and at the time of symptoms but prior to treatment. The swabs were immediately stored in RNeasy Lysis Buffer (Qiagen) after collection and stored at –20°C for a week and then at –80°C until RNA extraction. To isolate total RNA, the cells were rinsed from the swabs with ice-cold PBS, incubated with acid phenol, and lysed by bead beating. The nucleic acids were purified chloroform-isoamyl alcohol and ethanol precipitated. The samples were treated with the TURBO DNA-free DNase Treatment kit (Ambion), after which RNA was purified using the RNeasy Mini Kit. Samples are enriched for mRNA using a combination of the Ribo-Zero rRNA Removal Kits for gram-negative, gram-positive, and human/mouse/rat RNA (Epicentre Biotechnologies). Absence of the DNA contamination in these samples was verified by PCR using 16S rRNA primers. The clinical study protocol was approved by the Institutional Review Board of the University of Alabama at Birmingham and the University of Maryland School of Medicine. Written informed consent was appropriately obtained from all participants.

RNA-seq and gene expression analysis

All RNA-seq libraries (non-strand-specific, paired end) were prepared with the TruSeq RNA Sample Prep kit (Illumina). The total RNA samples isolated from the in vitro infections and the murine kidneys were subject to poly(A) enrichment as part of the TruSeq protocol. One hundred nucleotides of sequence was determined from each end of each cDNA fragment using the HiSeq platform (Illumina) per the manufacturer's protocol. Sequencing reads were annotated and aligned to the Ensembl GRCh38 (Cunningham et al. 2015) of the human reference genome, the Ensembl GRCm38 of the mouse reference genome, or the *C. albicans* reference genomes (SC5314 or WO-1) using TopHat2 (Kim et al. 2013). The alignment files from TopHat2 were input to Cufflinks to calculate the gene expression for each gene in each sample and differential expression across samples was determined using Cuffdiff (Trapnell et al. 2012). For the differential expression analysis of host gene expression, we took a more conservative approach and combined the RNA-seq data from the infections with SC5314 and WO-1, for each host cell type, to increase the statistical power. Both *C. albicans* strains were endocytosed at similar rates in each of

the host cell types (Supplemental Fig. S1). For differential expression of the *C. albicans* genes, the data for the different strains and host cell types were kept separate. A gene was considered differentially expressed if the false discovery rate (FDR) for differential expression was <0.05 .

qRT-PCR analysis

Prior to performing real-time PCR, cDNA was prepared using the RETROscript reverse transcriptase kit (Life Technologies). Real-time PCR was performed in triplicate in optical 96-well plates using the Power SYBRGreen PCR Master (Life Technologies). The primers used are listed in Supplemental Table S11. The mRNA levels of NEDD9- and PDGF BB-target genes were normalized against the internal *GAPDH* mRNA control using the $\Delta\Delta C_t$ method (Kubista et al. 2006).

Identification of signal transduction pathways

We used the Upstream Regulator Analytic of IPA (Ingenuity Systems; <http://www.ingenuity.com>) to identify signaling proteins that are potentially activated or repressed during the course of infection. This software assesses the overlap between experimentally derived gene lists and an extensively curated database of target genes for each of several hundred known regulatory proteins. It then uses the statistical significance of the overlap and the direction of the differential gene expression to make predictions about activation or repression of these regulatory proteins. The database has target lists for individual proteins as well as for protein complexes (Fig. 2; Supplemental Tables S6, S8).

PDGFRB and NEDD9 siRNA

HUVECs and OKF6/TERT-2 cells were grown to 80% confluence in six-well plates and then transfected with siRNA for *PDGFRB* (Thermo Fisher Scientific) and *NEDD9* (Qiagen) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Control cells were transfected with random control siRNA (Qiagen) in parallel. After 72 h post-transfection, the extent of *PDGFRB* and *NEDD9* knockdown was monitored by immunoblotting of whole-cell lysates with anti-PDGFRB antibodies (Clontech: Cat. # 22B1 for HUVECs; Cell Signaling: Cat. # C82A3 for OKF6/TERT-2 cells) and anti-NEDD9 antibodies (Cell Signaling: Cat. # 2G9). At least 80% knockdown was achieved in all experiments.

Measurement of host cell endocytosis

The endocytosis of *C. albicans* cells by HUVECs and OKF6/TERT-2 cells was determined by our standard differential fluorescence assay as described previously (Park et al. 2005; Phan et al. 2007). Briefly, the host cells were grown on fibronectin-coated glass coverslips and infected with 10^5 cells of *C. albicans* in RPMI 1640 medium. After a 90-min (HUVECs) or 180-min (OKF6/TERT-2 cells) incubation, the cells were rinsed twice with PBS and then fixed with 3% paraformaldehyde. The nonendocytosed organisms were stained with an anti-*C. albicans* rabbit serum (Biodesign International) conjugated with Alexa 568 (Molecular Probes). Afterward, the cells were rinsed extensively with PBS and then permeabilized with 0.05% (vol/vol) Triton X-100 in PBS. Next, the cell-associated organisms (the endocytosed plus nonendocytosed organisms) were stained with the anti-*C. albicans* rabbit serum conjugated with Alexa 488 (Molecular Probes). The coverslips were observed with an epifluorescence microscope. The number of organisms endocytosed by the host cells was determined by subtracting the number of cell-associated organisms (labeled with Alexa

568, which fluoresces red) from the total number of organisms (labeled with Alexa 488, which fluoresces green). At least 100 organisms were counted on each coverslip, and all experiments were performed in triplicate.

In some experiments, the host cells were incubated with the PDGFR inhibitors, AG1296 (Selleck Chemicals) (Kovalenko et al. 1997), and PDGFR Tyrosine Kinase Inhibitor III (CAS 205254-94-0, Santa Cruz Biotechnology) (Matsuno et al. 2002) at a final concentration of 10 μ M. The inhibitors were added to the host cells 1 h before the *C. albicans* and were present for the duration of the experiment. Control cells were exposed to an equal concentration of the diluent (0.2% DMSO).

Data access

All of the raw sequencing reads from this study have been submitted to the NCBI Sequence Read Archive (SRA; <http://www.ncbi.nlm.nih.gov/sra>) under accession numbers SRP011085 and SRP028748. All of the raw and processed gene expression data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE56093.

Competing interest statement

S.G.F. is a shareholder in and consultant for NovaDigm Therapeutics, Inc.

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