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MG289 in *Mycoplasma genitalium* Enhances Microbial Invasion and Bacterial Persistence in Benign Human Prostate Cells*

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ABSTRACT

Introduction: Recent studies suggest that infectious organisms may facilitate initiation and metastasis of many human cancers. One infectious organism of interest is *Mycoplasma genitalium* (Mg), a prevalent organism in humans known to cause sexually transmitted infection, as well as urethritis and prostatitis. Previous studies have demonstrated that benign, non-tumorigenic human prostate cells (BPH-1) chronically exposed to *M. genitalium* led to the malignant transformation of these cells as demonstrated in *in vitro* and *in vivo* models. Based on work from our laboratory, we felt this malignant transformation revolved around a specific *M. genitalium*'s ABC transporter (MG289) with homology to *M. hyorhinis*' ABC transporter, p37. In this study, differences in *M. genitalium*'s ability to infect and induce a unique proteome conducive to tumoral growth were studied with engineered *M. genitalium* in which the p37 protein was silent. **Materials and Methods:** Wild-type *M. genitalium* (strain 431c, designated as *M. genitalium* WT) and MG289 deficient *M. genitalium* mutant (strain 260_3, designated as Mg260_3) were used for this study. We studied the infectivity potential between *M. genitalium* WT and Mg260_3 upon exposure to BPH-1 cells. Furthermore, we set out to identify a unique proteome in BPH-1 cells exposed to *M. genitalium* WT that could explain its ability to induce malignant transformation of benign cells. Validation of selected proteomic targets was carried out by Western blot analysis. **Results:** Both *M. genitalium* WT and Mg260_3 strains showed somewhat similar growth curve when absorbance at 450nm was matched at day 0. Colony forming units (CFUs) were similar for both strains at the same absorbance. However, the ability to infect BPH-1 cells was greatly reduced in Mg260_3 compared to the *M. genitalium* WT ($p < 0.001$). This was evident by infectivity assays and confocal microscopy. Proteomic analysis of BPH-1 cells infected with either *M. genitalium* WT or Mg260_3 for 8 hr, 24 hr and 6 days demonstrated a considerable shift in protein expression over uninfected BPH-1 cells at each time point. The preponderance of perturbed proteins regulated protein synthesis and protein processing, triggering endoplasmic reticulum stress. **Conclusions:** In summary, we demonstrate that Mg260_3, which is deficient of the phosphonate ABC transporter substrate-binding protein; MG289 (homologue to *M. hyorhinis* p37), is less effective in invading and maintaining an intracellular persistence in benign human prostate cells. In addition, deletion of MG289 resulted in altered BPH-1 responses to *M. genitalium* infection as evidenced by differential proteome profiling of BPH-1 infected cells.

Keywords: Cancer; ER Stress Proteins; Infection; *Mycoplasma genitalium*; Prostate; Proteomics

1. Introduction

Mycoplasma genitalium (Mg) is the smallest known self-replicating microorganism capable of living in parasitic association with host cells, thus obtaining most of their nutrients that they cannot synthesize from the host for survival. *M. genitalium* can grow in close relationship with mammalian cells, often without any apparent pathology or symptoms for extended periods of time [1]. *M. genitalium* has been implicated in urogenital infections of both men and women around the world and it is recognized as an emerging cause of sexually transmitted infec-

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tions in the United States [2]. Specifically, *M. genitalium* has been identified as an independent etiologic agent of acute and persistent male non-gonococcal urethritis (NGU) and the cause for approximately 20% - 35% of non-chlamydial NGU cases [3,4]. In addition, because of the urethra's intimate location within the prostate gland, *M. genitalium* has also been linked with chronic persistent prostatitis [5,6].

Several lines of evidence indicate a strong association between mycoplasmal infection and various cancers. Genomic instability, such as polyploidy, trisomy, and translocations, has been reported in eukaryotic cells infected with mycoplasmas [7-9]. For example, *M. fermentans* and *M. penetrans*, which are found in high frequencies in patients suffering from AIDS, can induce permanent karyotypic changes in mouse embryo C3H cells and murine myeloid 32D cells [9,10]. *M. fermentans* infection of human peripheral blood mononuclear cells can also promote immortalization of these cells in culture [11]. The p37 gene of *M. hyorhinis*, also called Cypl (extracytoplasmic thiamine binding lipoprotein, phosphonate ABC transporter substrate-binding protein), has been implicated to induce a more aggressive cellular phenotype (*i.e.*, increase invasive ability) in FS9 mouse sarcoma cells [12]. It is believed that phosphonate ABC transporter substrate-binding protein from *M. hyorhinis* disrupts cellular contact of FS9 cells from fibroblasts, thereby making them more motile and invasive [12,13]. Blocking *M. hyorhinis* phosphonate ABC transporter substrate-binding protein with antibodies directed against phosphonate ABC transporter substrate-binding protein or removing mycoplasma infection reduces the invasive potential evident in mammalian cells exposed to *M. hyorhinis* or *M. hyorhinis* phosphonate ABC transporter substrate-binding protein [12,13].

Furthermore, recombinant *M. hyorhinis* phosphonate ABC transporter substrate-binding protein was shown to enhance the invasive potential of the human prostate cancer cells PC-3 and DU145, as well as the human melanoma cancer cells C8161 and A375M, while the enhancement in invasion could be abrogated by a specific neutralizing antibody directed at *M. hyorhinis* phosphonate ABC transporter substrate-binding protein [14]. Moreover, PC-3 and DU145 prostate carcinoma cells that were chronically exposed to recombinant *M. hyorhinis* phosphonate ABC transporter substrate-binding protein exhibited nuclear enlargement and increased migratory potential that coincided with significant changes in the expression of genes involved in cell cycle, signal transduction and metabolism [15]. Lastly after 19 weeks in culture, *M. hyorhinis* and *M. genitalium* infected human benign prostate cells, BPH-1, demonstrated malignant transformation evident in a panel of *in vitro* assays and confirmed by the formation of xenograft tumors in athymic mice. Associated with these changes was an increase in karyotypic entropy, evident by the accumulation of chromosomal aberrations and polysomy [8]. Interestingly, phosphonate ABC transporter substrate-binding protein from *M. hyorhinis* has significant homology with phosphonate ABC transporter substrate-binding protein from *M. genitalium* (MG289) [16].

Based on these findings illustrating, the importance of the mycoplasmal phosphonate ABC transporter substrate-binding proteins, we hypothesized that inactivation of *M. genitalium* phosphonate ABC transporter substrate-binding protein would have dire consequences in the ability of mycoplasma to exert its effect on benign human cells. In the present study, we investigated the pathogenic potential of *M. genitalium* phosphonate ABC transporter substrate-binding prostate (MG289) on BPH-1 cells by using a MG289 deficient mutant of *M. genitalium* (Mg260_3) that was originally created by global transposon mutagenesis and compared it in a panel of *in vitro* assays to wild-type Mg (Mg WT) [17]. In concert with previous findings [17], loss of the MG289 protein did not alter *in vitro* growth characteristics of Mg260_3 when compared to *M. genitalium* WT. However, this mutation caused a significant reduction in the ability of *M. genitalium* to adhere to and invade BPH-1 cells. Quantitative proteome studies of BPH-1 cells infected with Mg260_3 or *M. genitalium* WT also revealed that loss of the *M. genitalium* phosphonate ABC transporter substrate-binding significantly altered how BPH-1 cells responded to mycoplasmal infection. Namely, BPH-1 cells infected with *M. genitalium* WT exhibited significant changes in processes that involve regulation of gene expression and repair, and protein processing and modification. Furthermore, BPH-1 cells infected with *M. genitalium* WT exhibited a different and more robust endoplasmic reticulum stress response than uninfected cells or cells infected with Mg260_3 that if chronically present could incite and sustain the growth of human cells.

2. Materials and Methods

2.1. Cell Culture

Human benign prostate cell line, BPH-1 was obtained from the American Type Cell Culture (Manassas, VA). Cells were maintained in RPMI media supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. All cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 in air. All culture media were purchased from Invitrogen (Carlsbad, CA). BPH-1 cells previously tested negative for a panel of mycoplasmal organisms by PCR (Mycoplasma Detection Ste from Takara, Japan) was used for the study. Cells also tested negative for Mg and Mh by standard PCR. Primers for Mg: forward primer-5'-GGTCATG

GACAAACAAATTCAGCTTCAC-3'; reverse primer-5'-CAATACCAATGTCATACAAAAGCGGATCA-3' and Mh: forward primer-5'-CGATCCAACTGTTCAAGGTAATTTTTA-3'; reverse primer-5'-CACTTTTTCTAAACACTCCAACATCAT-3' were used. PCR was carried out using 20 µl reaction volume containing 200 ng of DNA and 200 nM of primers (Promega PCR mix). The amplification conditions of PCR consisted of initial denaturation at 95˚C for 2 min followed by 40 cycles of denaturation at 95˚C for 45 sec, annealing at 55˚C for 45 sec and elongation at 72˚C for 45 sec min, followed by 1 cycle at 72˚C for 10 min.

2.2. Mycoplasma Preparation and Culture

Stock solutions of both *M. genitalium* strains were prepared by growing the microorganisms to early log phase in SP4 broth, and stored frozen at −80˚C. Both strains of *M. genitalium* were grown in SP4 media under standard conditions [8].

2.3. Growth Curve

Cultures for colony forming unit (CFU) determination of inoculum were serially diluted 10-fold to 10-8 in SP4 broth then 20 ml from each sample and its corresponding dilutions were plated in SP4 broth and Agar plates. Broth cultures were incubated at 37˚C in ambient air, while Agar plates were incubated at 37˚C in 5% CO_2. Broth tubes were checked daily for a color change, and the reciprocal of the last dilution to show growth was deemed the color-changing unit (CCU). In order to confirm growth of *M. genitalium* WT and Mg260_3, CCU, which provide a relative indication of the amount of microbes present, were determined. Samples were serially diluted tenfold to 10-8 in SP4 broth in duplicate. Broth tubes were checked daily for color change, and the CCU was recorded as the reciprocal of the last dilution to show growth at 14 days. Agar cultures were incubated for at least 14 days before colonies were counted. Log-phase cultures of *M. genitalium* WT and Mg260_3 with the same absorbances (*i.e.*, same number of colonies) at 450 nm were diluted to 20 ml to give zero absorbance at 450 nm compared to SP4 blank on day 0 and growth was observed over a period of 14 days. Absorbance was read every 24 hrs and plotted graphically. The same absorbances gave same CFUs for both *M. genitalium* WT and Mg260_3 cultures. The experiment was carried out in duplicate in three separate experiments and p value of 0.05 was considered significant.

2.4. Infectivity Assay

BPH-1 cells (10^5) were inoculated with log-phase cultures (3×10^4 CFU) of *M. genitalium* WT or Mg260_3 to determine whether the mycoplasmal protein MG289 altered its ability to attach to and infect human cells [18]. To quantify intracellular *M. genitalium* load, the inoculum was removed following 3 hrs incubation for cellular attachment and entry and replaced with medium containing gentamicin (200 µg/ml, Sigma) for 2 hrs. Infected cells were washed thoroughly and fresh RPMI media added to the wells. The ability for *M. genitalium* to persist in culture media and not be transported into human cells was also quantified from culture supernatants in separate wells. Cell fractions or culture supernatants were collected at 8 hrs, 24 hrs and 6 days post-infection following removal of the inoculums for quantification of bacterial loads using both CCU and CFU assays. In every case, intracellular *M. genitalium* was quantitated by Real-Time PCR (RT-PCR) and standard PCR using MG289 and p110 primers. Extracellular *M. genitalium* was observed and quantitated using CFU assay ($p < 0.05$; Student's t-test). Each assay was performed in duplicate.

2.5. Real-Time PCR/ PCR

Following infections, DNA was extracted from cells using DNA isolation kit (Qiagen) as per manufacturer's instructions. Quantitative reverse transcriptase (RT)-PCR was carried out using ABI 7300 Real-Time PCR System (Life Technologies) in a 20 µl reaction volume containing 1 µl of the DNA, 1 µM of gene-specific TaqMan primer and probe mix. The primers used were purchased from Integrated DNA Technologies. Mg p110: Probe-5'-/56-FAM/AGCGAGCAGCATGACCAACTTGTT/36-TamSp/-3'; Primer 1-5'-TGAACCGGAAAGTGCAACAAGTGC-3' and Primer 2-5'-AAACTTCCCTGCATTGCTGTTCCC-3'. Human GAPDH: Probe-/56-FAM/AAAGCCAGTCCCCAGAACCCC/36-TAMSp/-3'; Primer 1-5'-CTCCCACCTTTCTCATCCAAG-3' and Primer 2-5'-ACATCACCCCTCTACCTCC-3'. Relative fold changes in DNA levels were calculated after normalization to GAPDH using the comparative Ct method (27). PCR was also carried out using 20 µl reaction volumes containing 200 ng of DNA, 1 µM of gene-specific TaqMan primer and probe mix for semi-quantitative analysis. Standard DNA PCR was carried out for *M. genitalium* MG289 using primers described earlier and Mg p110 primers-forward primers-5'-AAGGGCAAAACGCAAAGG GA-3' and p110-reverse primers-5'-TCCCCCTCCAGAAACA CTGG-3' for validation of *M. genitalium* WT and Mg260_3.

2.6. iTRAQ/LC-MS/MS

BPH-1 cells (10^6) were plated and allowed to attach and grow for a day. Next, cells were infected with log-phase *M. genitalium* WT or Mg260_3 for 3 hrs (initial infection time, 3×10^5 bacteria per infection), followed by 2 hrs

incubation with 200 μg/ml gentamicin in fresh media. Cells were then washed three times with PBS to remove unbound or non-invading mycoplasma. Fresh media was added and the cells were grown for a total of 8 hrs, 24 hrs or 6 days post initial infection time. Cells were washed with PBS again and displaced by versene, collected and centrifugation at 1500 × g for 5 min at 4˚C. Infection of all BPH-1 cell pellets was confirmed by PCR using MG289 and p110 primers as already described. Cell pellets were stored at −80˚C until processing for proteome studies. Protein lysates from cell pellets were prepared using mammalian proteome extraction kit (Calbiochem, Billerica, MA) according to the manufacturer's protocol.

Extracted protein samples were purified and concentrated using the Ultrafree®-0.5 centrifugal filter device (5 kDa cut-off from Millipore). Protein supernatants were dissolved in 1 M Urea/0.5M triethylammonium bicarbonate buffer and were assayed using CBX kit (from G-Biosciences). A total of three separate iTRAQ labelling reactions per infection were processed according to the manufacturer's instructions (AB Sciex) and as described by Alvarez *et al*. [19]. Briefly, the protein samples were reduced/alkylated and then digested with trypsin. Peptides were then labelled with iTRAQ reagents as follows; Uninfected 8 hrs, Uninfected 24 hrs and Uninfected 6 days with isobaric tag 114; *M. genitalium* WT 8 hrs, *M. genitalium* WT 24 hrs, *M. genitalium* WT 6 days with isobaric tag 115; Mg260_3 8 hrs, Mg260_3 24 hrs, Mg260_3 6 days with isobaric tag 116. For each iTRAQ experiment, peptides labelled with one of the three labels (114 - 116) were pooled before separation using strong cation exchange (SCX). The samples were dried and the peptides were separated using SCX as described by Alvarez [19]. Thirteen peptide fractions were collected, dried, and analyzed once by LC-MS/MS using nanoLC-ESI-LTQ Orbitrap Velos (Thermo Scientific) as previously outlined in [20]. The data acquired for the 13 fractions were processed and merged for each biological replicate independently.

The raw protein files were processed for peak detection using Mascot Distiller v2.4 (Matrix Science Inc., Boston, MA, USA). The MS/MS settings include single peak window selection from *m/z* 113.5 to *m/z* 117.5. The protein peak was identified and quantified with Mascot Daemon v2.3 (Matrix Science Inc.) using NCBInr (Jan 2012) entries for Bacteria (7,982,362 sequences) and Mammalia (811,620 sequences). The following settings were used for the search: trypsin as cleavage enzyme; two missed cleavages; methylthio modification of cysteines, iTRAQ (N terminal), iTRAQ (K) were fixed modifications; and methionine oxidation, deamidation (N, Q), and iTRAQ (Y) were selected as variable modifications. The mass error tolerance for precursor ions was set to 15 ppm and 0.08 Da for fragment ions. An automatic decoy database search was also performed to assess the false positive rate of protein identification. Automatic isotope correction was carried out using the values supplied with the AB Sciex reagents. For reporting quantification results, the protein ratio type used was the "weighted" geometric mean, normalization method was "summed intensities", outlier removal was "automatic" (Dixon's method up to 25 data points, Rosner's method above 25 data points), the peptide threshold was "above homology" (*i.e*., peptide score does not exceed absolute threshold but is an outlier from the quasi-normal distribution of random scores), and the minimum number of peptides for protein identification and quantification was two with an expected value <0.05. The intensity values of the assigned peptides were summed and the protein ratio calculated from the summed values.

2.7. Lysate Preparation and Western Blotting

Corresponding protein lysates from uninfected BPH-1 cells and cells infected with *M. genitalium* WT and Mg260_3 were prepared using mammalian proteome extraction kit (Calbiochem) as described earlier and 10 μg of protein was run on polyacrylamide-SDS gel. The proteins were transferred to a nitrocellulose membrane and immunoblotted with antibodies corresponding to various proteins identified from the proteomics. Blots were also probed for ER stress proteins using the ER stress Antibody kit (cat# 9956, Cell Signaling Technology, Danvers, MA). Mouse monoclonal antibody to *β*-Actin was purchased from Sigma-Aldrich, St. Louis, MO and used as a loading control.

2.8. Immunofluorescence and Confocal Microscopy

BPH-1 cells were plated onto cover slips (2×10^4 cells per cover slip) and were infected with log phase *M. genitalium* WT or Mg260_3 for 3 hrs followed by 200 μg/ml gentamicin treatment for 2 hrs. Cells were washed with PBS twice and then fixed with 10% buffered-formalin for 15 min. Uninfected cells were used as control. Immunofluorescence was performed as per the protocol published previously [21]. The primary *M. genitalium* antibody (polyclonal anti-rabbit Mg obtained from Dr. Mary B. Brown, University of Florida, College of Veterinary Medicine) was used at 1:1000 dilution. Secondary antibody was mouse anti-rabbit Alexa Fluor-488 (Molecular Probes, Carlsbad, CA). Control experiments (*i.e*., no primary *M. genitalium* antibody) demonstrated no cross-reactivity between mouse anti-rabbit secondary antibody as evident by no detectable staining by secondary antibody alone (data not shown). DAPI (Vector labs, Burlingame, CA) was used to stain the nuclei. Cells were visualized with a DM16000 inverted Zeiss scanning con-

focal microscope with a 63X/1.40NA oil immersion objective.

2.9. Data Analysis

M. genitalium's ability to invade human cells was analyzed by ANOVA followed by Tukey's test. A value of $p \leq 0.05$ was considered significant. Cluster analysis of iTRAQ data was performed as previously described [22] using JMP Genomics software (SAS, Cary, NC). Briefly, only protein ratios that were deemed to be significantly different from uninfected cells as determined by Pro GroupTM algorithm were used in the analysis. Protein ratios (*M. genitalium* infected/uninfected control) from each time point were normalized prior to clustering and clustering was performed by the method of Ward.

3. Results

3.1. Growth of *Mycoplasma genitalium*—Wild Type and Mutant Strains

Mycoplasma genitalium MG289 mutant clone (Mg260_3, *M. hyorhinis* p37 homologue deleted) was created by TN4001 transposon insertion in the MG289 gene; the transposon is located 570 bases into the 1158 bp MG289 gene [17]. Disruption of MG289 gene was checked by PCR (**Figure 1(A)**). *M. genitalium* WT was positive for both MG289 and p110 whereas Mg260_3 was positive for only p110 gene confirming that MG289 gene in Mg260_3 is disrupted. *In vitro* growth properties of *M. genitalium* WT and Mg260_3 were assessed in SP4 growth media by CCU method and optical density measurements taken at 450 nm (**Figure 1(B)**). During stationary phase, both *M. genitalium* WT and Mg260_3 attained similar microbial numbers. However, Mg260_3 did not attain stationary growth until day 7 of culture whereas *M. genitalium* WT achieved stationary phase by day 6 of culture (**Figure 1(B)**). Live bacteria were confirmed by CFU assay (data not shown).

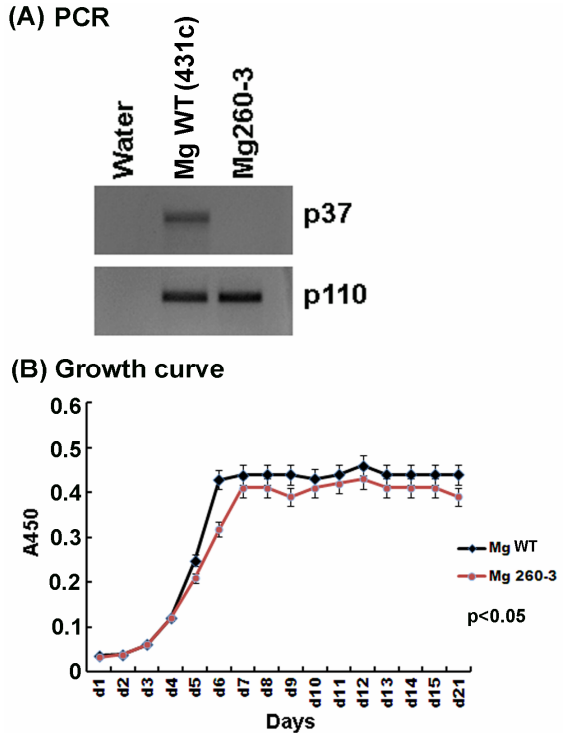

Figure 1. Growth curves of *M. genitalium* (*M. genitalium* WT and Mg260_3). (A) PCR confirming the depletion of MG289 (homologue to *M. hyorhinis* p37) in the mutant strain, Mg260_3; (B) Growth curve shows that *M. genitalium* WT reaches stationary phase earlier than Mg260_3 ($p < 0.05$). Values represent the mean and standard error of one experiment performed in triplicate. Each experiment was conducted three times.

3.2. *Mycoplasma genitalium* MG289 Facilitates Microbial Invasion and Persistence in BPH-1

Previous studies have shown that *M. genitalium* can invade and persist in endocervical cells [23]. Therefore, we evaluated the ability of *M. genitalium* WT and Mg260_3 to invade and persist in BPH-1 cells at 2 hrs, 8 hrs, 24 hrs, and 6 days post-inoculation. BPH-1 cell lysates collected at 2 hrs post-inoculation confirmed that bacteria attached and invaded BPH-1 cells. In follow-up assays assessing internalized bacteria, these BPH-1 cells exposed to *M. genitalium* WT and Mg260_3 and subsequently treated to remove extracellular *M. genitalium* had its DNA isolated and subjected to semi-quantitative PCR using a PCR primer/probe set specific for the p110 gene, which is present in both *M. genitalium* WT and Mg260_3 (**Figure 2(A)**). At 40 cycles, *M. genitalium* WT DNA was detected with equivalent intensity at all post-inoculation time points. This is in contrast to Mg260_3 in which less PCR product was detected at 2, 8, and 24 hrs post-inoculation. There was no amplified DNA detected in Mg260_3 infected cell lysates obtained from 6 day old cultures (**Figure 2(A)**).

We next quantified internalized *M. genitalium* bacteria by RT-PCR in BPH-1 cell RNA lysates obtained from a separate infectivity assay (**Figure 2(B)**). As shown in **Figure 2(B)**, significantly more *M. genitalium* WT were retrieved from BPH-1 cells at all post-infection time points compared to Mg260_3 ($p < 0.001$). Although there was a significant decline in the DNA copy number of both *M. genitalium* WT and Mg260_3 over the course of the experiment, the decline was more pronounced in Mg260_3 suggesting that deletion of MG289 may impact both microbial adherence and invasion. Viable bacteria were retrieved from both *M. genitalium* WT and Mg260_3 infected cultures (**Figure 2(C)**) confirming that bacteria that invaded BPH-1 cells were viable and that *M. genitalium*

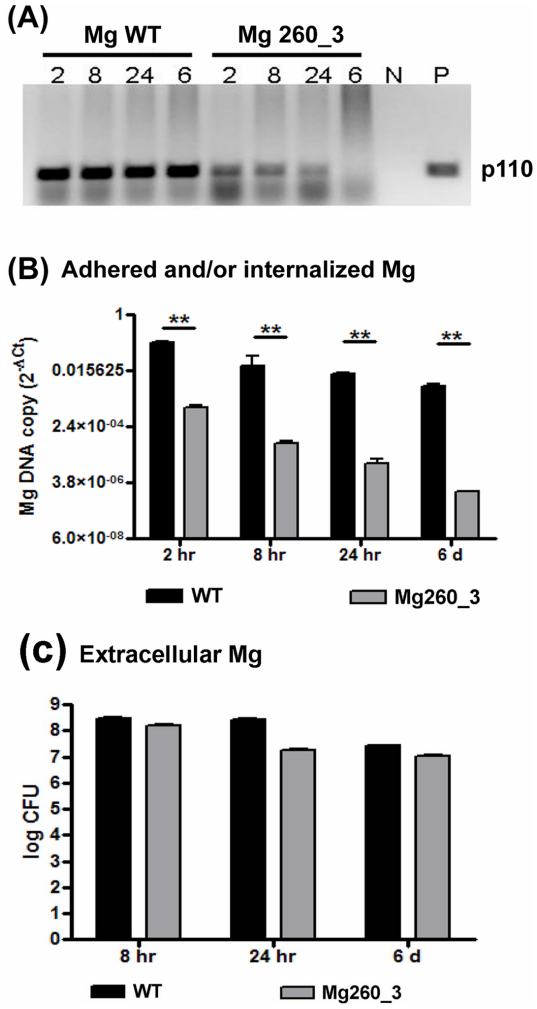

Figure 2. MG289 (homologue to *M. hyorhinis* p37) gene facilitates adherence and invasion of *M. genitalium* in BPH-1 cells. (A) PCR showing presence of *M. genitalium* within BPH-1 cells. *M. genitalium* WT could infect cells with higher efficiency than Mg260_3; (B) DNA copy number as determined by RT-PCR. *M. genitalium*g DNA was normalized to BPH-1 GAPDH DNA. **Indicates a significant difference between *M. genitalium* WT and Mg260_3 ($p < 0.05$); (C) Extracellular *M. genitalium* as determined by culture. Values represent the mean ± SD log CFU of triplicate. Each experiment was conducted three times.

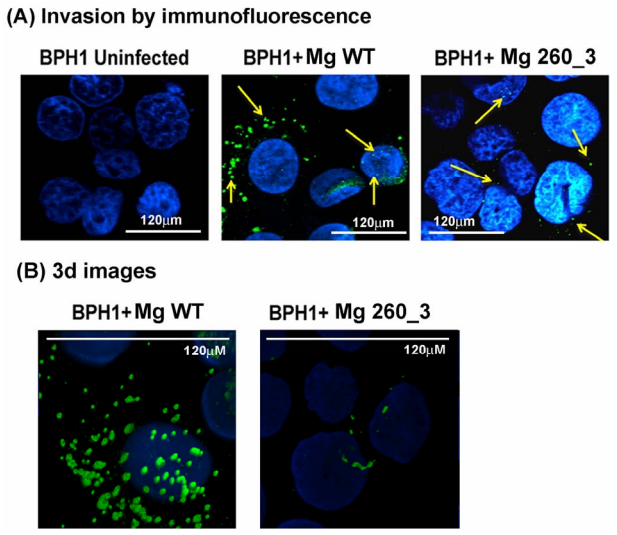

Figure 3. Intracellular location of *M. genitalium* within BPH-1 cells. (A) Representative confocal microscopic images of uninfected and *M. genitalium* infected (*M. genitalium* WT and Mg260_3) BPH-1 cells at 7 hrs post-inoculation. Images were captured using 63X/1.40NA oil immersion objective with DM16000 inverted Zeiss scanning confocal microscope, magnification −120 μm; (B) Images are 3 dimensional representation of infected cells, magnification −120 μm.

may be trafficking through BPH-1 cells in a similar fashion as human endocervical cells [23].

Infection of BPH-1 cells with *M. genitalium* WT and Mg260_3 was further examined by confocal microscopy (**Figure 3**). For infection experiments, all *M. genitalium* cultures were adjusted so that 10^5 BPH-1 cells were inoculated with 3×10^4 CFU of *M. genitalium* WT or Mg260_3 that were harvested during log phase growth. At 5 hrs post-inoculation, both internalized *M. genitalium* WT and Mg260_3 were found within the perinuclear region of BPH-1 cells by microscopy (**Figure 3(A)**), which is consistent with previous studies [24]. However, considerably more aggregates of *M. genitalium* WT were found within BPH-1 cells than Mg260_3. The staining pattern for *M. genitalium* was specific since neither antibody controls, nor uninfected BPH-1 cells were positive for *M. genitalium* immunofluorescence (**Figure 3(A)**, first panel). **Figure 3(B)** is a 3 dimensional representation of *M. genitalium* WT and Mg260_3 in BPH-1 cells.

3.3. BPH-1 Cells Infected with *Mycoplasma genitalium* Exhibit an Altered Proteome Profile

In order to identify the host cell processes that are perturbed by *M. genitalium* infection, we used quantitative proteome profiling of uninfected, *M. genitalium* WT infected and Mg260_3 infected BPH-1 whole cell extracts. The BPH-1 infection and cell harvest scheme is summarized in **Figure 4**. Proteins from BPH-1 cell extracts that were harvested at 8 hr, 24 hr, and 6 days post-inoculation were identified and quantified using Mascot v2.3. The Venn diagram (**Figure 5(A)**) illustrates the number of proteins that significantly changed in abundance in at least one of the ratios (*M. genitalium* WT *vs*. uninfected control, Mg260_3 *vs*. uninfected control or *M. genitalium* WT *vs*. Mg 260_3).

Next, we examined the BPH-1 proteome for proteins that may be affected during microbial invasion such as the endosome pathways [25,26], ubiquination and proteasome degradation pathways [27] and proteins involved

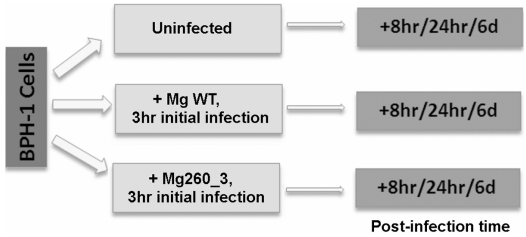

Figure 4. Diagram summarizing the proteome experimental design. BPH-1 cells were subjected to Mg infection (*M. genitalium* WT and Mg260_3) for an initial period of 3hr, followed by removal of the inoculum and gentamicin treatment (200 µg/ml) for 2hr to eliminate any remaining bacteria that were not internalized in BPH-1 cells. Infected BPH-1 cells were then harvested at 8 hr, 24 hr and 6 days post-inoculation and analyzed by PCR—to confirm presence of Mg, iTRAQ analysis—to identify proteome, and Western blot—to confirm targets from proteome.

in SRP-dependent co-translational protein targeting [28]. BPH-1 cells infected with *M. genitalium* WT exhibited a significant increase in endosome specific proteins and proteins that regulate endosome formation and trafficking. In contrast, the majority of these proteins were decreased in Mg 260_3 infected cells. *M. genitalium* infection also perturbed proteins involved in the proteasome degradation pathway and the SRP-dependent co-translational protein targeting to membrane pathway, which may have an impact on endoplasmic reticulum function [29]. The majority of proteins associated with these processes were significantly increased in BPH-1 cells infected with *M. genitalium* WT, but decreased in cells infected with Mg 260_3.

Two approaches were used to profile the effect of *M. genitalium* WT and Mg260_3 infection on BPH-1 cells. In the first analysis, protein ratios (*M. genitalium* WT/control and Mg260_3/control) that reflected a significant change ($p \leq 0.05$), and were accurately identified in all infected groups were used in this analysis (**Figure 5(B)**). Thus, proteins that did not conform to these criteria were removed before analysis. At 8 hrs post-inoculation, cluster groups 4 and 9 (demarcated with arrows) displayed the most dramatic differences. Proteins found within cluster group 4 were markedly reduced in *M. genitalium* WT infected cells when compared to cells infected with Mg260_3. Assigned functions of these proteins include oxidative reduction, perturbation induction of apoptosis, angiogenesis/cytokine activity/glycolysis, and negative regulation of cell proliferation. In contrast proteins in cluster group 9 were markedly increased in *M. genitalium* WT infected cells and their assigned functions include virus infectious cycle, cellular protein metabolic process, transcriptional regulation, and regulation of apoptosis.

Cluster analysis of cell cultures harvested at 24 hrs post-inoculation, revealed three clusters that were dramatically different between *M. genitalium* WT and Mg260_3 infected cells (**Figure 5(B)**). Proteins in clusters 4 and 6 were markedly increased in *M. genitalium* WT infected cells, and their assigned functions include apoptosis, cellular response to reactive oxygen species, biosynthetic processes, cell differentiation, regulation of microtubule function, protein import into the nucleus, mRNA processing, and inhibition of transforming growth factor receptor signaling pathway. Conversely, proteins in cluster 9 were markedly reduced in Mg WT infected cells, and these proteins are involved in transcription, translation, and inhibition of DNA damage response or signal transduction by p53 class mediator.

Cluster analysis of infected cultures harvested on Day 6 identified three markedly different cluster groups between *M. genitalium* WT and Mg260_3 infected cells (**Figure 5(B)**). BPH-1 proteins in cluster group 6 were markedly reduced compared to cells infected with Mg260_3, and these proteins are involved in translation, regulation of defense response, telomere maintenance, RNA processing, and steroid hormone mediated signaling. Proteins in clusters groups 9 and 10 were markedly increased in *M. genitalium* WT infected cells, and their assigned functions include regulation of cytokinesis, cell-cell signaling, vesicle-mediated transport, translation, and long-chain fatty acid biosynthetic process.

The biological response of BPH-1 cells to *M. genitalium* WT and Mg260_3 was also evaluated by determining the proportion of biological functions or cellular processes that were significantly perturbed by *M. genitalium* infection (**Figure 6**). Protein ratios that reflected a significant difference between *M. genitalium* WT and Mg260_3 infected cells ($p \leq 0.05$) were grouped according to their assigned biological process based on the blast2go gene ontology database (http://www.blast2go.com) [30]. Most notable changes were observed in proteins over the course of the assay involved in genetic processes such as regulation of transcription, RNA processing and DNA repair (20% - 22%). Similarly, another 20% of proteins that were significantly altered between Mg WT and Mg260_3 infected cells are involved in protein processing such as post-translational modification, enzyme activation, unfolded protein response, and protein targeting. Though the infectivity is suppressed in Mg260_3, absence of MG289 has produced significant changes in the proteome of infected BPH-1 cells (**Figure 6**).

3.4. Endoplasmic Reticulum (ER) Stress Response Increases with *Mycoplasma genitalium* Infection

It is believed that microbial pathogens may manipulate the ER stress response as a means of prolonging their intracellular survival within host cells [31]. Since Mg infection resulted in significant perturbations in the proteasome pathway, which can affect the ER stress response

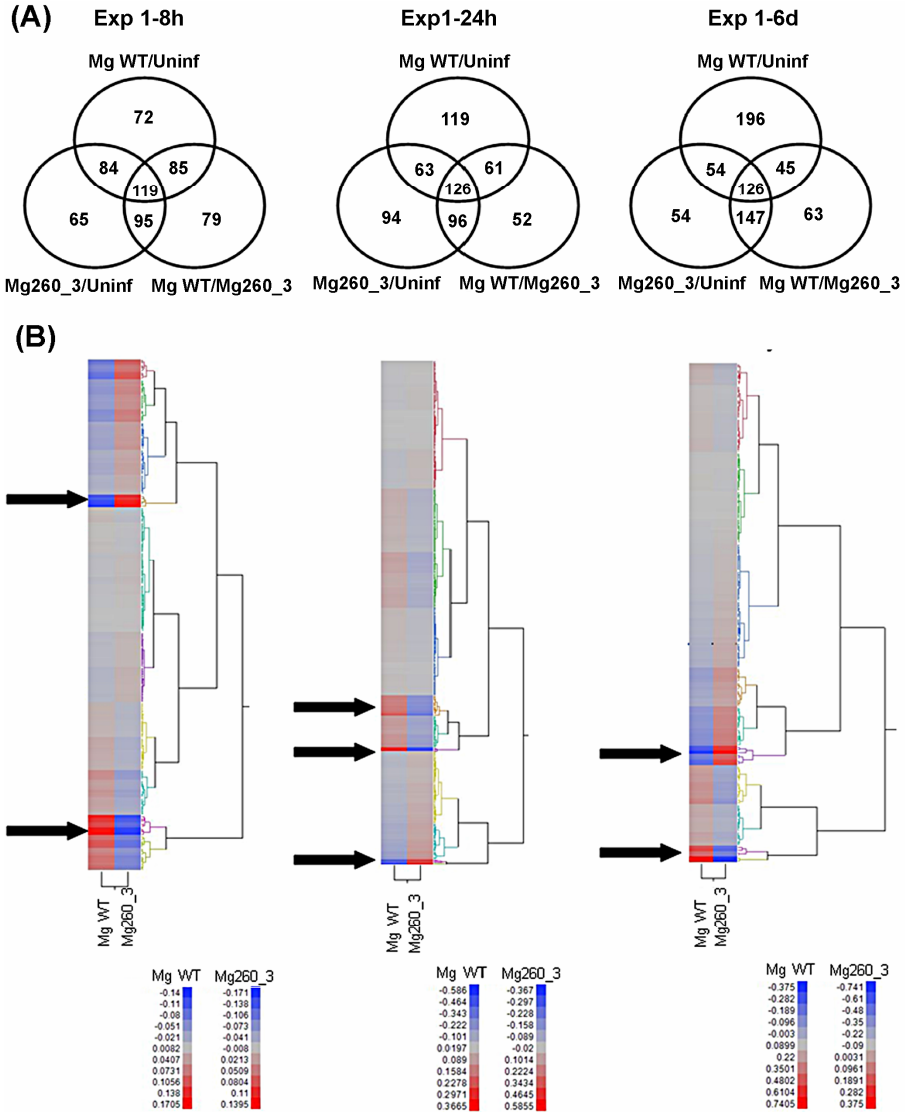

Figure 5. Proteomic analysis reveals a significant number of proteins changing in expression with *M. genitalium* WT infection/uninfected control and Mg260_3 infection/uninfected control. (A) Venn diagram illustrates the number of proteins that significantly changed in abundance in at least 1 of the ratios between comparisons (*M. genitalium* WT *vs.* uninfected control, Mg260_3 *vs.* uninfected and *M. genitalium* WT *vs.* Mg 260_3); (B) Heat maps were generated with protein ratios (*M. genitalium* WT/control and Mg260_3/control) that reflected a significant change ($p \leq 0.05$), and were accurately identified in all infected groups used in this analysis.

or unfolded protein response [32], we assessed the ER stress response in uninfected and *M. genitalium* infected BPH-1 cells by measuring a panel of ER associated proteins by Western blot analysis. We did not observe any changes in resident ER proteins such as calnexin, BiP, protein disulfide isomerase, and endoplasmic oxidoreductin-1 (data not shown). However, we did observe changes in proteins that regulate UPR [reviewed in Martin Hausmann, International Journal of Inflammation 2010] such as IRE1 (Inositol-requiring enzyme-1), PERK (protein kinase RNA-like endoplasmic reticulum kinase), and CHOP/GADD153 (CCAAT/-enhancer-binding protein homologous protein) (**Figures 7(A)** and **(B)**). At 8hr and 24 hr post-infection, both *M. genitalium* WT and Mg260_3 infected cells exhibited similar responses. Namely, both *M. genitalium* WT and Mg260_3 infection induced an increase in PERK with no appreciable change in the levels of IRE-1 and CHOP. However, by 6 days post-inoculation, *M. genitalium* WT infected cells had a marked increase in CHOP, whereas cells infected with Mg260_3 displayed a reduction in all three UPR proteins (**Figures 7(A)** and **(B)**).

4. Discussion

M. genitalium is an obligate parasite of the human uro-

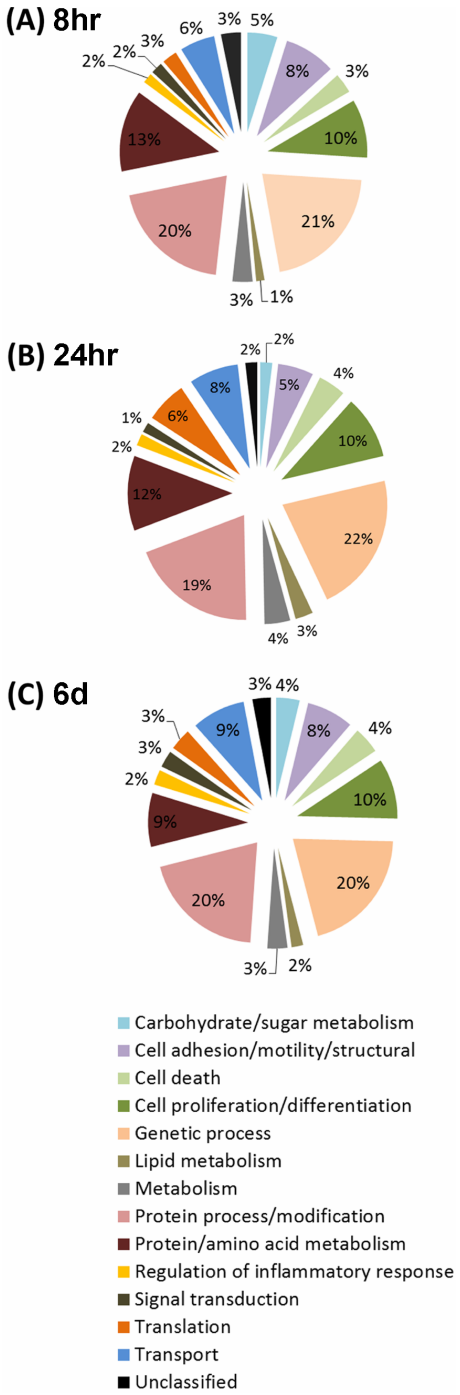

Figure 6. Functional distribution of protein ratios that were significantly different between *M. genitalium* WT and Mg260_3 infected cells at 8hr (A), 24 hr (B), and 6 d (C) post-infection. Gene ontology designations were obtained from the Blast2go database.

genital tract [33] that has been implicated in pelvic inflammatory disease [34], acute and persistent male nongonococcal urethritis (NGU) [3,4], and chronic persistent prostatitis [5,6]. Furthermore, *M. genitalium* may also play a role in cancer since our group has previously demonstrated that chronic exposure of human BPH-1 cells to *M. genitalium* resulted in malignant transformation of these benign cells as evident by *in vitro* and *in vivo* assays [8]. Despite the various reports indicating an association between Mg infection and human diseases, the means by which *M. genitalium* can persist in the host and thus contribute to the pathobiology of these diseases has not been determined.

In an attempt to identify the minimal essential genes for life, Glass *et al*. [17] established that the phosphonate ABC transporter substrate-binding protein, MG289 (homologue to Mh p37), in *M. genitalium* was not necessary for the survival of this microbe in cell free culture media. However, given that *M. genitalium* is an obligate parasite, retention of MG289 suggests that this gene serves a critical function during natural infection. Another interesting feature of MG289 is its similarity to the *M. hyorhinis* p37 [16], which has been shown to enhance malignancy in various cells lines [12-15]. Based on these characteristics, we postulated that MG289 serves an important role in the virulence of *M. genitalium*. In this report we have demonstrated that MG289 enhances microbial attachment, invasion and intracellular persistence in BPH-1 cells. Further, by using quantitative proteome profiling, we have been able to identify *M. genitalium* WT induced perturbations in BPH-1 cells that pertain to DNA repair, protein synthesis and protein processing, which could impact endoplasmic reticulum function.

Overall, our studies with *M. genitalium* WT were consistent with previous reports [17,18,24]. Although we observed a slight delay in the exponential growth phase of Mg260_3 in SP4 media, this mutant did attain a similar microbial density as *M. genitalium* WT at stationary phase, reaffirming that MG289 is not essential for growth of mycoplasma in cell free culture media [17]. We also noted that both *M. genitalium* WT and Mg260_3 could invade BPH-1 cells, and also exist outside of these cells. Furthermore, both internalized *M. genitalium* WT and Mg260_3 were in association with the nucleus of BPH-1 cells as well as in the cytoplasm. However, deletion of MG289 affected the efficiency with which *M. genitalium* could invade BPH-1 cells, and this may have also impacted the ability of the mutant to maintain an intracellular existence. For example, the temporal decline in the number of internalized Mg260_3 was more pronounced than with Mg WT, but the numbers of extracellular bacteria found within BPH-1 cultures were similar for both strains at all examined time points. *M. genitalium* WT and Mg260_3 also induced different proteome profiles pertaining to intracellular trafficking. For instance, BPH-1 cells infected with *M. genitalium* WT exhibited greater concentrations of proteins involved in endosome recycling and late endosome/lysosome pathways. Since we examine BPH-1 whole cell lysates, these differences in

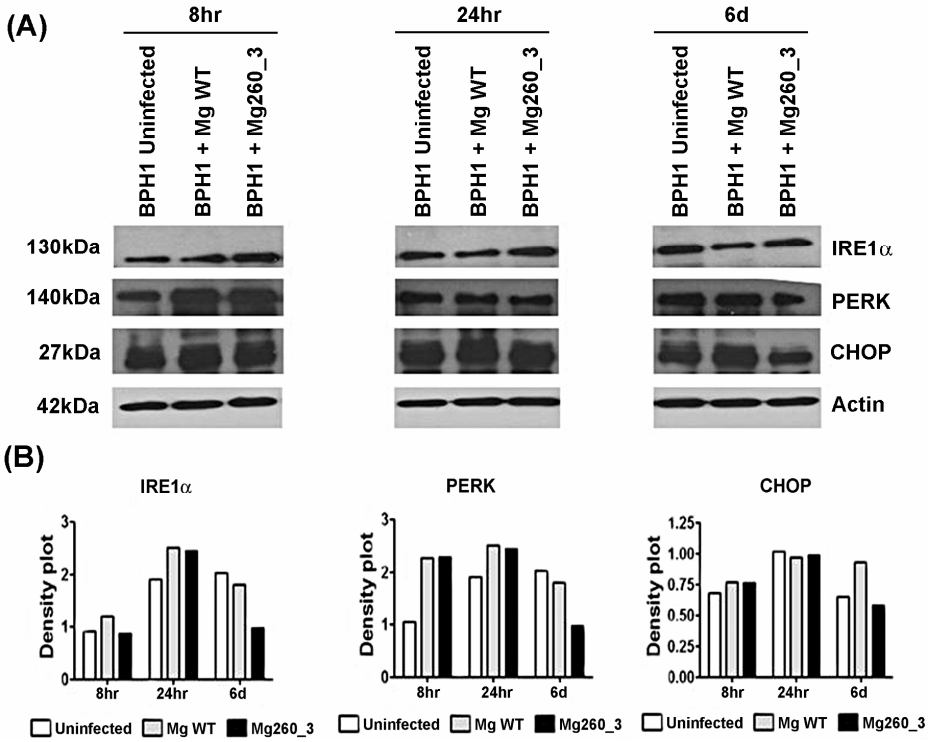

Figure 7. Western blot analysis reveals changes in protein expression at various time points with *M. genitalium* WT and Mg260_3 infections of BPH-1 cells. (A) Western blot analysis of ER stress proteins IRE1*α*, PERK and CHOP; (B) Western blot densitometry analysis of the above ER stress proteins IRE1*α*, PERK and CHOP show differences in expression between *M. genitalium* WT infected cells when compared to Mg260_3 infected BPH-1 cells.

protein abundance may be reflecting shifts within different cellular compartments (from cytoplasm to endosome), which are required in rapidly proliferating cells (*i.e*., cancer cells) [35,36].

There have been reports suggesting that *M. genitalium* localization to the nucleus may be important in microbial persistence within the host by evading both host defense and antibiotic mediated killing [37,38]. Intranuclear existence within host cells may also play a role in *M. genitalium* induced disease including malignant transformation [8]. Other bacterial pathogens can cause host cell DNA damage through their association with the host cell nucleus [39-41]. As already stated, we identified intracellular *M. genitalium* in association with BPH-1 nucleus, and this was most pronounced in Mg WT infected cells. In addition, our proteome studies in *M. genitalium* WT infected cells revealed significant differences in the abundance of proteins that regulate transcription, ubiquination, apoptosis, cell cycle, and DNA repair. These alterations may be a reflection of disturbed cell functional pathways that are associated with carcinogenesis and tumor progression [42,43]. Although our analysis was restricted to infections of less than one week in duration, the perturbations that we noted in our proteome analysis may be early indicators of how *M. genitalium* induces transformation of BPH-1 cells [8]. Longer-term experiments are currently underway.

The preponderance of perturbed proteins that regulate protein synthesis, protein processing, and their transport prompted us to consider that *M. genitalium* infection may be triggering endoplasmic reticulum stress [44]. Therefore, we screened uninfected and infected BPH-1 cells for indicators of a UPR by Western blot. Both *M. genitalium* WT and Mg260_3 induced a similar effect, which was an increase in UPR [45]. However, as the duration of infection progressed, the pattern of the UPR response changed between *M. genitalium* WT and Mg260_3. This suggests that the UPR is being triggered by other *M. genitalium* factors besides MG289, and that the quality of the response changes with duration of infection. The restoration of IRE1 and PERK levels coupled with an increase in CHOP in *M. genitalium* WT infected cells was an intriguing finding. This pattern may be an indication of a successful adaptive response by *M. genitalium* to an intracellular existence [46,47]. Indeed, manipulation of the host UPR by both viruses and bacteria have been reported, and they can lead to detrimental effects within the host [48-50]. For example, herpes simplex virus infection induces phosphorylation of PERK/GADD34 and downstream signaling involving eIF2*α*, which in turn facilitates viral production within host cells [48]. A number of studies on Human cytomegalovirus (HCMV) indicate

that HCMV maintains UPR responses that are beneficial to the virus while simultaneously inhibiting cellular processes that are detrimental to them [49]. For instance, binding of HCMV Pp28 to BiP/GRP78 maintains the integrity and function of the HCMV assembly compartment [51]. Infection of myeloid cells with *Chlamydia trachomatis* increases CHOP expression and assists in the production of pro-inflammatory cytokine, IL-23 [50]. Hence it is not surprising to find that *M. genitalium* can also induce an ER stress response, which may be important for its survival and extracellular persistence within the host.

Activation of UPR is also associated with infection-induced carcinogenesis [52,53]. Increased Hspa5 and Chop expression is specifically associated with *Helicobacter felis* induced gastric dysplasia in C57BL/6 mice [52]. Enhanced CHOP expression is associated with human papillomavirus (HR-HPV) infection and abnormal p53 expression in squamous cell carcinoma of uterine cervix [53]. Therefore, the increased expression of CHOP in *M. genitalium* WT infected cells, may also be associated with malignant transformation of BPH-1 cells during chronic exposure to *M. genitalium* [8].

This is the first study to confirm a pathogenic role for MG289 (*M. hyorhinis* p37 homologue in *M. genitalium*) in benign human prostate cells. We demonstrate that *M. genitalium* mutant, MG289, is capable of infecting BPH-1cells, though it exhibits a much-suppressed ability to infect when compared to *M. genitalium* WT. This feature can be exploited therapeutically to curb mycoplasma infection. Both *M. genitalium* WT and Mg260_3 are capable of altering the proteome profile of infected BPH-1 cells and the pathways that are altered may relate to oncogenic transformation. The differences in pathways related to DNA repair, endosome trafficking and UPR between the *M. genitalium* WT and Mg260_3 can further be explored in carcinogenesis as well as in the effective eradication of *M. genitalium* infections.

5. Conclusion

In summary, we demonstrate that Mg260_3, which is deficient in the phosphonate ABC transporter substrate-binding protein; MG289 (homologue to *M. hyorhinis* p37), is less effective in invading and maintaining an intracellular persistence in benign human prostate cells. In addition, deletion of MG289 resulted in altered BPH-1 responses to *M. genitalium* infection as evidenced by differential proteome profiling of BPH-1 infected cells.

6. Authors Contributions

All authors have read and approved the final manuscript.

WR—Performed experiments, Acquisition of data, drafting of manuscript.

LR—Interpretation of proteomics data, drafting of manuscript.

JK—Performed Western blot assays.

SD—Study concept and design, revision of manuscript.

CJR—Study concept and design, supervision, acquisition of funding and drafting of manuscript.

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