International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Transcriptome modulation of bovine trophoblast cells in vitro by *Neospora caninum*

Pilar Horcajo [a], Laura Jiménez-Pelayo [a], Marta García-Sánchez [a], Javier Regidor-Cerrillo [a], Esther Collantes-Fernández [a], Daniel Rozas [a,1], Nina Hambruch [b], Christiane Pfarrer [b], Luis Miguel Ortega-Mora [a,*]

[a] SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[b] Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany

ARTICLE INFO

Article history:
Received 9 May 2017
Received in revised form 31 July 2017
Accepted 19 August 2017
Available online xxxx

Keywords:
Neospora caninum
Bovine trophoblast
Isolates
Virulence
Transcriptome

ABSTRACT

Neospora caninum is one of the most efficient transplacentally transmitted pathogens in cattle and is a cause of abortion in this domestic species. The invasion and proliferation of *Neospora caninum* in the placenta and its dissemination to the foetus are crucial events in the outcome of an infection. In the bovine placenta, the placentomes are formed by maternal caruncles, which are delimited by a maternal epithelium and foetal cotyledons, which are delimited by an epithelial layer named the trophoblast. These epithelia form a physical barrier against foetal infection. Furthermore, trophoblast cells act as an innate immune defence at the foetal-maternal interface. *Neospora caninum* invades and proliferates in trophoblast cells in vitro, but it is unknown whether host cell modulation events, which affect the immune response and other processes in the trophoblast, occur. In this work, we investigated the transcriptomic modulation by *Neospora caninum* infection in the bovine trophoblast cell line F3. In addition, two *Neospora caninum* isolates with marked differences in virulence, Nc-Spain1H and the Nc-Spain7, were used in this study to investigate the influence of these isolates in F3 modulation. The results showed a clear influence on extracellular matrix reorganisation, cholesterol biosynthesis and the transcription factor AP-1 network. Interestingly, although differences in the transcriptome profiles induced by each isolate were observed, specific isolate-modulated processes were not identified, suggesting very similar regulation in both isolates. Differential expression of the *N. caninum* genes between both isolates was also investigated. Genes involved in host cell attachment and invasion (SAG-related and microneme proteins), glideosome, rhoptries, metabolic processes, cell cycle and stress response were differentially expressed between the isolates, which could explain their variability. This study provides a global view of *Neospora caninum* interactions with bovine trophoblast cells and of the intra-specific differences between two *Neospora caninum* isolates with biological differences.

1. Introduction

Neosporosis, which is caused by the protozoan parasite *Neospora caninum*, is a globally distributed disease that causes abortion in bovines and important economic losses to the cattle industry (Dubey et al., 2007; Reichel et al., 2013). Cattle can become horizontally infected following the ingestion of oocysts shed by a definitive host (several species of domestic and wild canids). More frequently, the infection is transmitted transplacentally from the infected dam to her foetus during pregnancy (Dubey et al., 2007). *Neospora caninum* is one of the most effective transplacentally transmitted pathogens. Parasite invasion of and proliferation in the placental tissues and dissemination to the foetus are crucial events in the outcome of *N. caninum* infections.

In the bovine placenta, the main sites of foetal-maternal interaction are the placentomes. These structures are formed by maternal caruncles, which are delimited by a maternal epithelium and foetal cotyledons, which are delimited by an epithelial layer named the trophoblast (Haeger et al., 2016). These cells produce a large variety of signalling molecules such as bovine placental lactogen and pregnancy-associated glycoproteins (Wooding and Beckers,

* Corresponding author at: Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.
E-mail address: luis.ortega@ucm.es (L.M. Ortega-Mora).
[1] Present address: Hologic Iberia SLU, Calle de Tarragona 161, 08014 Barcelona, Spain.

http://dx.doi.org/10.1016/j.ijpara.2017.08.007
0020-7519/

1987; Touzard et al., 2013). In addition, they participate in the synthesis of steroids and prostaglandins (Ullmann and Reimers, 1989; Matamoros et al., 1994), thus playing an important role in embryo implantation and pregnancy outcomes. Furthermore, trophoblast cells form the first barrier against foetal infection and act as an innate immune defence at the foetal-maternal interface, producing cytokines and chemokines in response to pathogenic infections, which can induce abortion (Entrican, 2002).

The interaction of *N. caninum* with bovine target cells remains largely unknown. Recently, we used the well-established bovine trophoblast cell line F3 to study the infection of two different isolates of *N. caninum*, the highly virulent isolate Nc-Spain7 and the comparatively avirulent isolate Nc-Spain1H (Jimenéz-Pelayo et al., in press). Both isolates grow in F3 trophoblasts, although they show significant differences in their adhesion, invasion, proliferation and egression, which could be related to the marked differences in virulence observed between these isolates in vivo. The Nc-Spain7 isolate causes abortion in experimentally infected pregnant heifers at 70 days gestation (Regidor-Cerrillo et al., 2014), whereas the foetus survives an infection by the low virulence isolate Nc-Spain1H (Rojo-Montejo et al., 2009). The molecular basis for the differences in the pathogenesis of neosporosis and the potential role of the differences in the modulation of the host cell by diverse *N. caninum* isolates remain largely unexplored. Studies carried out in the closely related parasite *Toxoplasma gondii* have shown its capacity to modulate different functions in the target host cells (Blader and Koshy, 2014; Hakimi and Bougdour, 2015). In addition, *T. gondii* strain-specific regulated host pathways have also been described (Melo et al., 2013; Croken et al., 2014). To address this gap in knowledge on *N. caninum*, we used the RNA-Seq technology to investigate how *N. caninum* modulates the transcriptome of bovine trophoblast cells and to discern whether the avirulent isolate Nc-Spain1H and the virulent isolate Nc-Spain7 differ in their regulation of trophoblast cells, which could explain in part the differences observed in pathogenicity.

2. Materials and methods

2.1. Parasites, cell cultures and experimental design

Tachyzoites from Nc-Spain7 and Nc-Spain1H isolates were routinely maintained in a monolayer culture of the MARC-145 cell line as previously described (Regidor-Cerrillo et al., 2011). The bovine trophoblast cell line F3 (Hambruch et al., 2010) was maintained in DMEM/Ham's F12 (Life Technologies, New York, USA) containing 10% FCS, 100 IU/ml of penicillin, 100 mg/ml of streptomycin and 2 mM glutamine.

F3 infections for transcriptomic analyses were performed as previously described (Jiménez-Pelayo et al., in press). T25 flasks with F3 monolayers were infected with a selected multiplicity of infection of eight for Nc-Spain7 and 10 for Nc-Spain1H in order to obtain the highest number of infected cells and a similar number of infected cells in both isolates, according to previous results (Jiménez-Pelayo et al., in press). Three additional T25 flasks with F3 monolayers were maintained under the same conditions, but those were not infected. Infected and non-infected cells were washed 4 h p.i. to remove non-adhered/non-invading tachyzoites. At 8 h p.i., when parasites had invaded, but had not commenced multiplication (Jiménez-Pelayo et al., in press), the cells and parasites were recovered by cell scraping in 5 ml of PBS, pelleted by centrifugation at 1350*g* for 10 min, resuspended in 300 µl of RNAlater (Thermo Fisher Scientific, Madrid, Spain) and stored at −80 °C until RNA extraction. All analyses were performed with three biological replicates.

2.2. RNA extraction

Total RNA from the culture samples was extracted using the Maxwell® 16 LEV simplyRNA Purification Kit (Promega, Madison, CA, USA), following the manufacturer's instructions. The RNA concentration and purity were assessed spectrophotometrically at 260/280 nm of absorbance using the NanoPhotometer® Classic (Implen, München, Germany). RNA integrity was determined by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualised under UV light. Only samples with total RNA extracts between 0.5 and 5 µg in concentration, those without signs of degradation, and with 260/280 ratios between 1.8 and 2.0, were included in the study.

2.3. Quality control of total RNA, library preparation and RNA-Seq data

The quality and quantity of the total RNA was determined in a Bioanalyzer 2100 and a Qubit 2.0. B (all samples had an RNA Integrity Number (RIN) between 9.6 and 10).

The poly(A)+ mRNA fraction was isolated from the total RNA and cDNA libraries were obtained following Illumina's recommendations. Briefly, poly(A)+ RNA was isolated on poly-T oligo-attached magnetic beads and chemically fragmented prior to reverse transcription and cDNA generation. The cDNA fragments then went through an end repair process, the addition of a single 'A' base to the 3′ end and then ligation of the adapters. Finally, the products were purified and enriched using PCR to create the final indexed double-stranded cDNA library. The quality of the libraries was then analysed in a Bioanalyzer 2100, High Sensitivity assay; the quantity of the libraries was determined by real-time PCR in a LightCycler 480 (Roche Applied Science, Mannheim, Germany).

Prior to cluster generation in cBot (Illumina, San Diego, USA), equimolar pooling of the libraries was performed. The pool of the cDNA libraries was sequenced by paired-end sequencing (100 bp ×2) in an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA, USA).

2.4. Computational analysis of RNA-Seq data

Quality control of the raw data were performed using the FastQC tool. Then, the raw paired-end reads were mapped against the *Bos taurus* version UDM3.1 genome provided by the Ensembl database using the Tophat2 algorithm (Kim et al., 2013). The unmapped reads were mapped against the *N. caninum* genome (http://toxodb.org/toxo/, ToxoDB-25) using Tophat2. Low quality reads (Phred score <10) were eliminated using the Picard Tools software, version 1.129 (http://picard.sourceforge.net/). Gene predictions were estimated using the Cufflinks method (Trapnell et al., 2010).

To ensure that the biological replicates are well correlated, principal component analysis (PCA) was performed using the adegenet library (Jombart, 2008) of the statistical software package R (http://www.r-project.org).

Differential expression analysis between the conditions was performed using the cuffdiff algorithm (Trapnell et al., 2013). Finally, we selected the differentially expressed genes with a *P* value adjusted by false discovery rate (FDR) of less than 0.05 and an absolute fold change (FC) of at least 1.5.

2.5. Functional enrichment and network analysis

For *B. taurus*, a hypergeometric test (Rice, 2007) was applied using the human orthologue obtained from the *B. taurus* annotation. We selected the functional categories with *P* values adjusted by FDR of less than 0.05. The outcome of the Gene Ontology (GO)

Please cite this article in press as: Horcajo, P., et al. Transcriptome modulation of bovine trophoblast cells in vitro by *Neospora caninum*. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.08.007