Characterization of *Campylobacter Rectus* Infection in Vitro and Its Effects on

Mouse Placental Function in Vivo

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Abstract

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Placental Function in Vivo

(Under the direction of Steven Offenbacher, DDS, PhD, MMSc; Silvana Barros, DDS, PhD

and Deborah Threadgill, PhD)

Campylobacter rectus is as a possible cause in the association between periodontal disease and adverse pregnancy outcomes. In this study we evaluated the effect of a distant infection with *Campylobacter rectus* on the fetal placental unit at the murine gestational day E14.5 and characterized the in vitro interactions of *C. rectus* with cells similar to those found in the placenta, ie. trophoblast and endothelial cells. Scarce infection with *C. rectus* was directly visualized within the placentas of challenged mice, which presented with decreased placental weight and fetal-placental unit weight and altered placental morphology. *C. rectus* infection dysregulated angiogenic gene expression in the placenta and in trophoblast murine cells in vitro. *C. rectus* was also capable of invasion of human trophoblast and endothelial cells while was only able to penetrate cell junctions in murine trophoblasts. The results illustrate the potential for *C. rectus* to affect placental/fetal development in mice and humans.

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Table of Contents

List of Tables

List of Figures

Chapter 1

Original manuscript: *Campylobacter rectus* translocates to the placenta of pregnant mice, induces changes in placental development and dysregulates placental gene expression.

Campylobacter rectus translocates to the placenta of pregnant mice, induces changes in placental development and dysregulates placental gene expression

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Abstract

The mechanisms behind the association between periodontal infection and adverse pregnancy outcomes are not completely understood. **Aims:** In this study we evaluated the effect of a distant infection with *Campylobacter rectus* on the fetal placental unit at the murine gestational day E14.5. Placental morphologic changes and levels of expression of 6 genes potentially important for placental development were measured. The presence of *C. rectus* in placental tissues was also evaluated. **Methods:** Timed pregnant BALB/c mice were challenged with *C. rectus* at gestational day E7.5. Animals were sacrificed at E14.5 and placental tissues were collected. Expression of *Vegf*, *Pgf*, *Flt-1*, *Vash1*, *Gal* and *Galr3* was measured by real time PCR. Presence of *C. rectus* in the placenta of pregnant mice was determined by immunostaining. **Results:** Mean placental weight and mean fetal-placental unit weight were lower in *C. rectus*-challenged mice. Low weight fetuses from challenged mice presented morphological differences in their placentas including a trend for an increase in the relative area of the decidua layer and infiltration of spongiotrophoblasts into the

2

labyrinth. Moreover, scarce infection with *C. rectus* was directly visualized within the placentas of challenged mice. While a positive correlation was observed between levels of expression of the four angiogenesis-related genes tested and fetal weight in control mice, *C. rectus* infection abolished this relationship. Similarly, *Vegf* and *Pgf* levels correlated positively with placental efficiency only in the control mice. Moreover, a negative correlation was seen between levels of expression of the neuropeptide galanin and fetal weight in challenged mice. In conclusion, a distant infection with *C. rectus* alters placental morphology and patterns of expression of genes important for placental/fetal development. This effect could be in part attributable to the direct presence of the bacteria within the placental tissues.

Introduction

In recent years, several studies have indicated that maternal chronic periodontal infection may be an independent contributor to abnormal pregnancy outcomes, including preterm births (birth that occurs at less than 37 weeks of gestation), low birth weight (defined as less than 2,500 grams) and preeclampsia (Boggess *et al.* 2003; Offenbacher *et al.* 1996; Offenbacher *et al.* 2001). Furthermore, it seems that not only presence of the disease, but disease progression during pregnancy could be a predictor of the severe adverse pregnancy outcome of very preterm (less than 32 weeks) birth, independently of traditional obstetric, periodontal, and social domain risk factors (Offenbacher *et al.* 2006). The potential mechanisms underlying these associations are still under investigation. Periodontal pathogens are capable of invasion of periodontal tissues and translocation to distant sites (Champagne *et al.* 2000), potentially activating a systemic inflammatory response that impairs fetal

development and initiates preterm delivery. Indeed, fetal exposure with *Campylobacter rectus* has been demonstrated to be associated with preterm neonates (Madianos *et al.* 2001). *C. rectus* is a gram-negative, microaerophilic and motile bacterium, part of the orange complex bacteria (Socransky *et al.* 1998) that has been found to be associated with the initiation and progression of periodontal disease (Ihara *et al.* 2003; Tanner *et al.* 1998). Other *Campylobacter* spp., *C. fetus* and *C. jejuni,* have been reported to be associated with miscarriage, premature labor and severe perinatal infection (Simor *et al.* 1986; Wong *et al.* 1990). A murine model of intra-chamber challenge with *C. rectus* has been developed (Yeo *et al.* 2005) to investigate the association between a distant infection with *C. rectus* and adverse pregnancy outcomes. This model demonstrated that remote subcutaneous maternal infection increases fetal resorptions and fetal growth restriction as measured at gestational day 16.5. A follow-up study also revealed that *C. rectus* challenge results in abnormal placental architecture at the same stage of development (Offenbacher *et al.* 2005). Typically, placentas from growth-restricted fetuses of challenged mice show decreased width of the vascular labyrinth and an increase in the decidual tissue and trophoblast layer, as compared to unchallenged dams. Challenged mice also show areas of trophoblast invasion of the decidua and vasodilation of the fetal placental circulation in the junctional zone. Focal areas of inflammatory infiltrate are also seen in challenged placentas. Use of the same model, also demonstrated that maternal infection results in *C. rectus* translocation to the placenta, as demonstrated by nested polymerase chain reaction (PCR) detection of *C. rectus* in 46.7% of the placental tissues from growth-restricted fetuses (Bobetsis *et al.* 2007). Direct contact of *C. rectus* with placental cells may induce inflammatory changes. The main cellular component of the placenta, trophoblast cells, has been shown to act as a pregnancy-specific

component of the immune system (Guleria and Pollard 2000) and to express toll-like receptors TLR-2 and TLR-4, with the induction of cytokine production after TLR-4 activation and the induction of apoptosis after TLR-2 engagement (Abrahams *et al.* 2004). Using the *C. rectus* murine model, Bobetsis *et al*. (Bobetsis 2006) carried out a microarray comparison of placentas from unchallenged and growth-restricted fetuses. It was found that several genes, known to be important for fetus development, were downregulated in the placentas from growth-restricted fetuses. Among these genes were the angiogenesis-related genes placental growth factor (*Pgf*) (1.56-fold) and vasohibin 1 (*Vash1*) (1.89-fold). Other genes downregulated in the placentas of growth-restricted fetuses were galanin (*Gal*) (2.38-fold) and one of its receptors galanin receptor 3 (*Galr3*) (1.72-fold). Close regulation of angiogenesis by VEGF-related genes might have fundamental effects on placental development (Stepan *et al.* 2006). Galanin is a neuropeptide that is expressed in placental tissues and may play an important role in the regional regulation of blood flow, critical for fetal growth and development. (Graf *et al.* 1996; Kleine *et al.* 2001; Zamlynski *et al.* 2007). In this study we evaluated the effect of a distant infection with *C. rectus* on the fetal placental unit at gestational day (E14.5), an earlier time point than that studied before (Offenbacher *et al.* 2005; Yeo *et al.* 2005). Morphologic placental changes and levels of expression of 6 genes potentially important for placental development were evaluated. These genes were *Vegfa*, *Pgf*, and their receptor *Flt-1*; the Vegfa-inducible anti-angiogenic gene *Vash1* and the vasoactive neuropetide *Gal* and its receptor *Galr3*. The presence of *C. rectus* in placental tissues was also evaluated by immunostaining.

Materials and Methods

5

Mouse Infection Model

All procedures were in accordance with the animal welfare guidelines and approved by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee. The mouse infection model used was similar to that described before (Offenbacher *et al.* 2005; Yeo *et al.* 2005). BALB/c mice were housed under controlled and standardized conditions, with 12-hour light-dark cycles. Regular mouse diet and water were provided *ad libitum*. Females were enrolled in the experiments at approximately 6 weeks of age, at which time a steel chamber was implanted subcutaneously (Lin *et al.* 2003b). After a period of healing, females were mated overnight with males of the same strain. The next morning, females were removed from the male cages and examined for vaginal plugs. If a plug was found, that day was recorded as embryonic day E0.5. Pregnant mice received an intrachamber injection of 100 μl of 10^9 CFU/mL live *C. rectus* or PBS at E7.5. Mice were sacrificed at E14.5 and fetuses (n=15 from 3 unchallenged dams and n=25 from 4 challenged dams) and their respective placental tissues were collected. Placentas and fetuses were weighted and values for control and challenged mice were compared using the unpaired Student's t test.

Bacterial Culture

Campylobacter rectus strain 314 was cultured under anaerobic conditions at 37° C on PRAS ETSA plates (Anaerobe Systems AS-546). Bacterial colonies were collected and used for challenges after 5 days of plate inoculation.

Placental Histology

For histological analysis placentas were collected and bisected sagitally then fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6 μm) were stained using standard hematoxylin and eosin protocol. Morphometry was performed on 8 placentas from control dams and 9 from *C. rectus* challenged mice, measuring three histological sections in each sample. Images were acquired using a Nikon Microphot-FXA Microscope equipped with a QImaging Micropublisher CCD camera for digital image acquisition. A 1X planapo na 0.04 and a 2X plan apo na 0.08 lense were used for visualization and acquisition of images for morphometric measurements. For identification of inflammatory cells within samples 40X Apo na 0.95 and 20X Apo na 0.75 lenses were used. The area occupied by each placental layer, namely decidua, spongiotrophoblast layer and labyrinth, was calculated using the "Image J" software (http://rsb.info.nih.gov/ij/). The area of each layer relative to the total placental area was expressed as percentage. Regression analysis was used to examine the relationship between fetal weight and the relative area of each placental layer using fetal weight as the outcome variable.

Detection of C. rectus in placental tissues

Placentas from 2 control mice (n placentas =10) and 2 challenged mice (n placentas =11) were examined for the presence of *C. rectus* by immuno-staining. Briefly, paraffin embedded sections were placed in a solvent to remove paraffin, re-hydrated by placement in

ethanol/H_2O washes and permeabilized by incubation in 0.2% Triton X in PBS. Slides were then incubated for 1 h in blocking buffer (5% BSA, 1% goat serum and 0.2% Triton-X) and then incubated overnight at 4ºC with a FITC-conjugated anti-*Campylobacter* antibody (Kirkegaard & Perry Laboratories) and Texas red-conjugated phalloidin (Invitrogen). Slides were washed several times in PBS and visualized with a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope and 60X na 1.4 planapo and 100X na 1.3 plan neo fluar lenses. Images were captured using the LSM510 META software.

RNA isolation and real-time PCR

Hemisected placentas from 2 control mice (n placentas = 15) and 2 challenged mice (n placentas = 25) were used for RNA isolation and measurement of gene expression. Placentas were homogenized and RNA was isolated following the Trizol protocol (Invitrogen). RNA pellets were dissolved in RNAse-free water and RNA concentration was determined using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was also checked by running samples in a Bioanalyser 2100 instrument (Agilent Technologies). Real-time PCR relative quantification was performed using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 1 µg RNA was reverse transcribed using the Omniscript RT kit (Qiagen, Valencia, CA), 10 µM random primers (Invitrogen) and 10 U RNase inhibitor (Ambion). Reactions were incubated at 37ºC for 1 h. One µl of this reaction was added to a real time PCR reaction containing one of the following primers and probe assay mixtures: Vascular endothelial growth factor A (*Vegfa*) (Mm00437304_m1), FMS-like tyrosine kinase 1 (*Flt1*)

8

(Mm00438980_m1), vasohibin 1 (*Vash1*) (Mm00616592_m1), galanin receptor 3 (*Galr3*) (Mm00443617_m1), galanin (*Gal*) (Mm00439056_m1), placental growth factor (*Pgf*) (Mm00435613_m1). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (rodent *Gapdh* control reagents) and hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) (Mm01545399_m1) were used as housekeeping genes. The comparative C_T method was used to calculate the relative quantification according to Applied Biosystems ABI Prism 7700 Sequence Detection System User bulletin #2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Briefly, the ΔC_T was determined by subtracting the average C_T value of the housekeeping gene from the average C_T value of the target gene. Then the $\Delta\Delta C_T$ for each gene was calculated by subtracting the mean ΔC_T of the controls from the ΔC_T of the challenged. The range for each gene relative to the controls was determined by evaluating the expression $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T +S$ and $\Delta\Delta C_T -S$, where S is the standard deviation of the $\Delta\Delta C_T$ value. Regression analysis was used to test for a relationship between fetal and placental weight and gene expression. Differences in parameter estimates between challenged and control groups where assessed with an interaction test.

Results

Campylobacter rectus infection affects the development of the fetal-placental unit at day E14.5

Table 1.1 shows a summary of the mice used in this study. Despite commonly seen variability among dams, mice infected with *C. rectus* had a lower mean placental weight and lower mean fetal-placental unit weight than the control mice (Table 1.2). Mean fetal weight was also lower in the challenged mice but this difference did not reach statistical significance.

Morphometric changes in the placenta of challenged mice

Placental layers were measured and their relative areas (% of the total placental area) were compared (Table 1.3). No difference in the mean percentage area for each placental layer was observed between challenged and control mice. Relative placental layer areas were also analysed for a correlation with fetal weight (Table 1.4). In control mice, no correlation was observed between the relative area of the different layers and fetal weight. In challenged mice, however, there was a trend for the relative size of the decidua layer to inversely correlate with fetal weight (p=0.08) which corresponded with the observation that those fetuses with the lowest weight had placentas with enlarged deciduas (Figure 1.1). Figure 1.2 depicts a placenta from a control mouse (1.2A) and a placenta from a low weight fetus of a challenged mouse (1.2B). Notice the increased size of the decidua layer in the challenged placenta and the infiltration of the labyrinth by cells from the spongiotrophoblast layer. We also observed inflammatory cell infiltration in challenged placentas as is depicted in Figure 1.2C-F.

Invasion of placental tissues by Campylobacter rectus

C. rectus was seen in the placentas of challenged mice but not in the placentas of control mice. *C. rectus* was detected in the placentas of 7 out of 11 challenged placentas stained. The presence of bacterial cells was not observed in the majority of the fields scanned but bacteria were rather scarcely distributed (Figure 1.3). Bacterial cells were always seen as single cells or as two cells together in a typical *C. rectus* "butterfly" arrangement (1.3C). Due to the scarce distribution of bacterial cells within placental tissues quantitation was not possible.

Effect of Campylobacter rectus on placental gene expression

The expression of 6 genes important for fetal/placental development was assayed in the placentas of challenged and control mice. Table 1.5 shows that no significant differences were seen in the expression of the genes analysed. Regression analysis was used to evaluate the relationship between fetal weight, placental weight, placental unit weight and placental efficiency (each as the outcome variable) and levels of gene expression (Tables 1.6-1.9). Placental efficiency was defined as the percentage of fetal weight of the total weight of the fetal-placental unit. As can be seen from the regression analysis, *C. rectus* infection was seen to affect the relationship between fetal weight and the levels of expression of the angiogenesis-related genes *Vegf, Pgf* and *Vash1* (Table 1.6). In control mice, levels of expression for these three genes in the placenta correlated positively with fetal weight, while

in challenged mice, levels of expression and fetal weight do not correlate thus fetuses with higher fetal weight do not necessarily have placentas with higher levels of expression and vice versa. A similar trend was also observed for the gene *Flt1*, the receptor for *Pgf* and *Vegf*, although the regression analysis did not reach statistical significance. Also, *Vegf, Flt1, Pgf* and *Vash1* expression in controls correlated positively with the total fetal placental unit weight (Table 1.8), a result mostly driven by the effect of gene expression on fetal weight as gene expression did not affect placental weight (Table 1.7). Interestingly, *Vegf* and *Pgf* expression also correlated positively with placental efficiency in control mice but *C. rectus* infection abolished this relationship (Table 1.9).

The levels of expression of the vasoactive neuropeptide galanin, on the contrary, correlated negatively with fetal weight in the challenged mice indicating that those mice with lower fetal weight (and possibly growth-restricted) had higher levels of galanin expression in their placentas (Table 1.6). A similar effect was seen when the levels of expression of galanin were correlated with fetal-placental unit weight (Table 1.8).

The levels of expression of the galanin receptor *Galr3* correlated positively with the weight of the fetal-placental unit in control mice. This is an effect that is probably the result of a nearly significant correlation between *Galr3* gene expression and placental weight and a less significant trend seen between *Galr3* gene expression and fetal weight. *C. rectus* infection also abolished this relationship.

Discussion

In recent years, evidence has emerged that a focal chronic infection such as periodontal disease can exert its effects on distant organs (Offenbacher *et al.* 1996; Paquette 2002; Paquette *et al.* 2007). Systemic dissemination of oral bacteria has been demonstrated, especially in patients with periodontal disease, in which ulceration of the sulcular epithelium is a common finding (Forner *et al.* 2006; Stelzel *et al.* 2002). Adverse pregnancy outcomes is among those systemic conditions associated with periodontal disease, however, the mechanisms behind the association are still under investigation. It is possible that individuals presenting both with periodontal disease and preterm delivery may have a genetic background that predisposes the individual to a hyperinflammatory response with excess secretion of proinflammatory mediators and cytokines. A hyperinflammatory phenotype may put those individuals simultaneously at risk for periodontal disease and abnormal pregnancy outcomes as inflammatory cascades are associated with both conditions (Gustafsson *et al.* 2006; Park *et al.* 2005). Another consideration is that periodontal pathogens, which are capable of invading periodontal tissues and translocating to distant sites (Champagne *et al.* 2000), activate a systemic inflammatory response. The latter could have an adverse effect on fetus development and initiate preterm delivery. These pathogens could potentially translocate from the oral cavity to the fetal-placental unit and have a direct effect on the expression of genes important for placental/fetal growth. Indeed, in a recent report (Barak *et al.* 2007) detected by PCR, the presence of 6 periodontal pathogens in placentas of both women with preeclamsia and healthy controls. Fifty per cent of the placenta specimens were

13

positive for one or more periodontopathogenic bacteria in the preeclampsia group, compared to only 14.3% from controls. Bacterial counts were also higher in the preeclampsia group for all of the periopathogenic bacteria examined. The present study confirmed, using a murine model, that periodontal bacteria such as *C. rectus* have the ability to translocate from a distant site of infection to placental tissues. Detection by PCR of *C. rectus* was previously reported in the same murine infection model, but at a later developmental time point (Bobetsis *et al.* 2007). *C. rectus* is capable of invasion of different cell types including human umbilical vein endothelial cells (HUVEC cell line) and human trophoblasts (BeWo cell line), an ability that might be important for translocation of the bacteria into the fetal-placental unit (Diaz *et. al*., manuscript in preparation).

Several animal models, mainly murine, have been employed to study the mechanism behind the association between periodontal organisms and adverse pregnancy outcomes (Han *et al.* 2004; Lin *et al.* 2003a; Lin *et al.* 2003b; Yeo *et al.* 2005). These investigations have revealed that *Porphyromonas gingivalis* infection introduced subcutaneously or via a periodontitis model, stimulates intrauterine fetal stress and impairs fetal growth (Lin *et al.* 2003a; Lin *et al.* 2003b). These changes were associated with maternal upregulation of tumor necrosis factor α and downregulation of the anti-inflammatory cytokine IL-10. It was also reported that following a challenge of *P. gingivalis* to pregnant mice, those litter mates that have placentas containing PCR-detectable *P. gingivalis* are greatly growth-restricted, whereas those with placentas negative for *P. gingivalis* are normal-sized or moderately growth-restricted (Lin *et al.* 2003b). Han *et. al.* (2005) developed a model with intravenous injection of *Fusobacterium nucleatum* to pregnant mice at day 17 of gestation. The injection resulted

in premature deliveries, still births and non-sustained live births. Interestingly, *F. nucleatum* had a specific tropism for the uterus, first internalizing into endothelial cells in the placenta and then invading the placental tissues, specifically the decidua layer, finally reaching the amniotic fluid. The murine model used in the present study was initially developed by Yeo *et al.* (2005). This model has demonstrated that remote subcutaneous maternal infection (initiated at day E7.5) increases fetal resorptions and fetal growth restriction as measured at day E16.5. The present study used the same model but mice were sacrificed at day E14.5 to examine if changes in placental development occur at an earlier gestational time point. Mean placental weight and the mean fetal weight were both lower in *C. rectus*-challenged mice. Analysis of the mean fetal weight, however, did not reach statistical significance. An explanation for this finding is the previous observation that the effect of challenge with *C. rectus* on fetal weight is clustered within dams, therefore, only some fetuses within a litter will suffer from growth restriction (Yeo *et al.* 2005). Clustering of the effect of *C. rectus* in challenged mice was also observed when we analyzed the relative area of the different placental layers. When the mean area for the 3 layers was compared between control and challenged mice, no differences were observed. However, regression analysis showed a trend for a correlation between fetal weight and area occupied by the decidua layer in challenged mice only, demonstrating that those mice with the lowest fetal weight had decidua layers of increased size. This observation was also present at day E16.5 (Bobetsis *et al.* 2007) indicating that changes induced by *C. rectus* infection in placentas of growth impaired-fetuses may persist throughout the different stages of pregnancy. An increase in the size of the decidua layer might reflect a maternal inflammatory response to protect the adjacent fetus from bacterial dissemination.

Essential requirements for successful gestation are the development of a functional placental vasculature together with the coordinated growth and differentiation of the placental tissues. Angiogenic VEGF family growth factors such as VEGF and PGF, as well as their receptors, are important for normal placental development (Lam *et al.* 2005). VEGF-A is the major player in angiogenesis among the VEGF family and activates two tyrosine-kinase receptors, VEGFR1 (FLT1) and VEGFR2 (KDR in humans/Flk-1 in mice) (Shibuya 2006). PGF is also a member of the VEGF family, with significant amino-acid homology to VEGF (Iyer *et al.* 2001) and shown to be expressed at high levels in human trophoblast cells and umbilical vein endothelial cells (Kleine *et al.* 2001). The FLT1 receptor also binds PGF and therefore is closely implicated in the regulation of angiogenesis by interacting with two main angiogenic growth factors (Torry *et al.* 2003). The role of FLT1 in regulating angiogenesis has been shown to be mostly inhibitory although FLT1 has potentially a dual role due to the fact that the FLT1 gene encodes for a full-length receptor form with transmembrane and extracellular domains and a short soluble form of the FLT1 protein that carries only the extracellular domain. The membrane bound receptor can act as a positive regulator of angiogenesis via its tyrosine kinase. On the contrary, the FLT1 soluble form can act as a decoy receptor via its ligand-binding domain that has the ability to bind VEGFa and PGF preventing their interaction with endogenous receptors (Shibuya 2001; Torry *et al.* 2003). Another gene closely related to VEGF is the recently described vasohibin 1. Vasohibin 1 is an endothelium-derived angiogenesis inhibitor that is induced by VEGF, therefore forming part of a negative regulatory loop (Sato and Sonoda 2007). Results from the present study show that infection with *C. rectus* abolishes the correlation present in the control mice between

levels of expression of angiogenesis-related genes and fetal weight. The levels of *Vegf* and *Pgf* were also seen to correlate with placental efficiency in control mice but not in mice infected with *C. rectus*. A study carried out using a pig model has shown that increases in placental VEGF mRNA are positively correlated with fetal weight and placental efficiency (defined as fetal weight/placental weight ratio) throughout different pregnancy stages (Vonnahme *et al.* 2001). A tight control of placental angiogenesis as the fetal demands increase will assure adequate capacity for nutrient transfer from the maternal to the fetal compartment. The concerted dysregulation of the correlation between fetal weight and angiogenesis-related genes by *C. rectus* infection could be related to the fetal abnormalities and growth restriction seen intra-uterus and after birth in challenged mice (Offenbacher *et al.* 2005).

We also studied the mRNA levels of the neuropeptide *Gal* and one of its receptors, *Galr3*. Galanin is a hypothalamic regulatory peptide involved in appetite regulation and reproduction. Galanin is secreted by different tissues including the nervous system, the anterior pituitary gland, adrenal medulla, pancreas, intestine and placenta (Graf *et al.* 1996; Zamlynski *et al.* 2007). Although the role of galanin in the mouse and human placenta has not been elucidated, we observed that in control mice there was a trend for the levels of galanin and its receptor *Galr3* to correlate positively with fetal weight and for the levels of the receptor only to correlate positively with placental weight. In the challenged placentas an inverse trend is observed where galanin levels correlate negatively with fetal weight and the relationship between fetal and placental weight and *Galr3* is abolished. The physiologic role of galanin in placenta and its relationship to fetal weight, however, is yet to be defined and

the interactions of galanin are complex as different receptors are recognized (Lang *et al.* 2007). One could speculate however, that if there is a positive relationship between galanin and fetal weight, the increased galanin levels seen in those challenged low weight fetuses could be a compensatory mechanism to account for the decreased fetal weight and/or the dysregulation in the expression of the receptor. To confirm that indeed the levels of expression of challenged low weight fetuses were higher than the levels for the control, the *Gal* levels from the 2 fetuses from each litter with the lowest fetal weight were compared to the pooled control *Gal* levels (data not shown). Indeed, this comparison indicated that low weight challenged fetuses have nearly 2-fold more *Gal* mRNA transcript levels than pooled controls. These results oppose those found at day E16.5 by Bobetsis (2006) indicating that perhaps we are seeing a transient compensatory regulation of galanin.

In summary, this study has provided further confirmation that a distant *C. rectus* infection induces placental abnormalities in mice. Moreover, *C. rectus* has the ability to translocate to placental tissues. Yet, the significance of the presence of the bacteria in the tissues is still questionable as a rather scarce number of cells were seen infecting the placenta and more quantitative studies need to be performed. We also report that infection results in changes in the relationship between placental gene expression and fetal weight that could have deleterious effects for the progression of fetal development.

Table 1.1. Summary data for control and challenged mice.

	Control			Challenged			
	Mouse #1	Mouse #2	Mouse #3	Mouse #1	Mouse #2	Mouse #3	Mouse #4
Mother's weight	31.169 g	29.100 g	29.600 g	28.338	30.300 g	30.300 g	28.900 g
No. pups per mother	6	4	5	4	7	7	7
No. resorptions	3	1	0	5	1	0	1
Mean fetal weight	0.130 g	0.163 g	0.144 g	0.105 g	0.160 g	0.156 g	0.095 g
Mean placental weight	0.123 g	0.110 g	0.119 g	0.108 g	0.088 g	0.101 g	0.122 g
Mean FW + PW[1]	0.253 g	0.253 g	0.264 g	0.213 g	0.248 g	0.257 g	0.217 g

[1] Mean FW + PW represents the weight of the fetal-placental unit.

Table 1.2. Comparison of fetal and placental weight in control and challenged mice

	Fetal weight	Placental weight	FW + PW
Mean control (n=15)	0.144±0.03	0.118±0.02	0.262±0.03
Mean challenged (n=25)	0.132±0.04	0.104±0.02	0.236±0.04
Student's t test (p)	0.272	**0.032**	**0.023**

Table 1.3. Morphometric measurements of the percentage of placenta area occupied by the

different placental layers in challenged and control mice

	Labyrinth	Spongiotrophoblast	Decidua
Control	46.2±17.5	23.1±15.4	30.7±12.6
Challenged	52.9±12.9	20.6±9.3	28.4±7.6

Table 1.4. Regression analysis of the correlation between percentage of area occupied by each placental layer and fetal weight

		Labyrinth	Spongiotrophoblast	Decidua	Junctional[1] (S + D)
All	R^2	0.02	0.05	0.04	0.01
	PE*	0.45	1.15	-0.58	-0.44
	P value	0.57	0.41	0.47	0.70
Challenged	R^2	0.28	0.20	0.42	0.33
	PE	2.29	3.08	-2.86	-3.34
	P value	0.14	0.22	0.08	0.14
Control	R^2	0.00	0.07	0.02	0.00
	PE	-0.04	-0.92	0.25	0.04
	P value	0.96	0.52	0.73	0.96

[1] Junctional zone includes both the decidua and spongiotrophoblast layers.

* PE: parameter estimate

Table 1.5. Real-time PCR relative quantification of gene expression in the placenta of mice challenged with *C. rectus* (n=2, n placentas= 15) and control mice (n=2, n placentas= 10)

Gene	*Gapdh*-normalized		*Hprt1*-normalized	
	Fold difference challenged/control	Range	Fold difference challenged/control	Range
Vegfa	1.21	0.54-2.67	1.01	0.53-1.93
Flt1	1.25	0.76-2.10	1.03	0.50-2.13
Pgf	1.52	0.67-3.45	1.38	0.80-2.39
Vash1	1.07	0.52-2.20	0.92	0.52-1.64
Gal	1.23	0.45-3.38	1.21	0.49-2.97
Galr3	1.14	0.30-4.29	0.98	0.37-2.61

Table 1.6. Regression analysis of the correlation between gene expression and fetal weight.

	All[1]			Control			Challenged			Slope Diff[2]
	R^2	PE	p-value	R^2	PE	p-value	R^2	PE	p-value	p-value
Vegf	0.078	12.728	0.21	**0.562**	**22.137**	**0.02**	0.014	6.708	0.70	0.45
Flt1	0.054	16.137	0.30	0.425	31.083	0.06	0.024	13.676	0.62	0.59
Pgf	0.094	13.538	0.17	**0.624**	**19.190**	**0.01**	0.044	19.566	0.49	0.99
Vash1	0.018	6.813	0.55	**0.481**	**23.281**	**0.04**	0.013	-6.954	0.71	0.19
Gal	0.006	-2.814	0.74	0.284	10.751	0.14	**0.331**	**-37.097**	**0.04**	**0.009**
Galr3	0.081	8.106	0.20	0.319	9.096	0.11	0.032	7.511	0.56	0.91

[1]Challenged and control mice gene expression levels and fetal weights were used for analysis.

[2]. Slope differences between challenged and control were evaluated by an interaction test.

Table 1.7. Regression analysis of the correlation between gene expression and placental weight.

	All[1]			Control			Challenged			Slope Diff[2]
	R^2	PE	p-value	R^2	PE	p-value	R^2	PE	p-value	p-value
Vegf	0.006	1.698	0.74	0.052	3.254	0.56	0.009	2.597	0.77	0.95
Flt1	0.000	-0.486	0.95	0.163	9.345	0.28	0.005	-3.168	0.81	0.45
Pgf	0.001	-0.522	0.92	0.072	3.165	0.49	0.001	-1.668	0.91	0.72
Vash1	0.016	3.202	0.58	0.052	3.726	0.55	0.016	3.803	0.68	0.99
Gal	0.001	-0.595	0.89	0.071	2.616	0.49	0.040	-6.343	0.51	0.36
Galr3	0.124	5.034	0.11	0.392	4.899	0.07	0.092	6.300	0.31	0.83

[1]Challenged and control mice gene expression levels and fetal weights were used for analysis.

[2]. Slope differences between challenged and control were evaluated by an interaction test.

Table 1.8. Regression analysis of the correlation between gene expression and fetal-placental unit weight.

	All[1]			Control			Challenged			Slope Diff[2]
	R^2	PE	p-value	R^2	PE	p-value	R^2	PE	p-value	P-value
Vegf	0.075	14.426	0.22	**0.504**	**25.291**	**0.03**	0.021	9.305	0.63	0.65
Flt1	0.038	15.652	0.39	**0.490**	**40.428**	**0.04**	0.011	10.509	0.73	0.94
Pgf	0.065	13.016	0.25	**0.576**	**22.355**	**0.02**	0.030	17.897	0.57	0.87
Vash1	0.029	10.015	0.45	**0.441**	**27.008**	**0.05**	0.002	-3.151	0.88	0.33
Gal	0.006	-3.410	0.73	0.299	13.366	0.13	**0.369**	**-43.440**	**0.03**	0.11
Galr3	0.159	13.140	0.07	**0.515**	**13.995**	**0.03**	0.087	13.811	0.33	0.85

[1]Challenged and control mice gene expression levels and fetal weights were used for analysis.

[2] Slope differences between challenged and control were evaluated by an interaction test.

Table 1.9. Regression analysis of the correlation between gene expression and placental efficiency (percentage of fetal weight of the total fetal/placental unit weight).

	All[1]			Control			Challenged			Slope Diff[2]
	R^2	PE	p-value	P-value	R^2	PE	p-value	R^2	PE	p-value
Vegf	0.094	3.016	0.17	**0.484**	**4.180**	**0.04**	0.028	2.158	0.58	0.65
Flt1	0.076	4.143	0.21	0.279	5.119	0.14	0.052	4.544	0.45	0.94
Pgf	0.102	3.060	0.15	**0.522**	**3.572**	**0.03**	0.046	4.517	0.48	0.87
Vash1	0.024	1.704	0.491	0.410	4.373	0.06	0.002	-0.659	0.88	0.33
Gal	0.000	0.047	0.98	0.220	1.923	0.20	0.119	-4.994	0.25	0.11
Galr3	0.024	0.0949	0.49	0.128	1.173	0.34	0.004	0.604	0.84	0.85

[1]Challenged and control mice gene expression levels and fetal weights were used for analysis.

Figure 1.1. Correlation between relative decidua area and fetal weight in challenged mice.

Correlation between relative decidua area and fetal weight in challenged mice

$$y = -2.8577x + 215.8$$
$$R^2 = 0.4239$$

Fetal weight (mg) vs % decidua area *
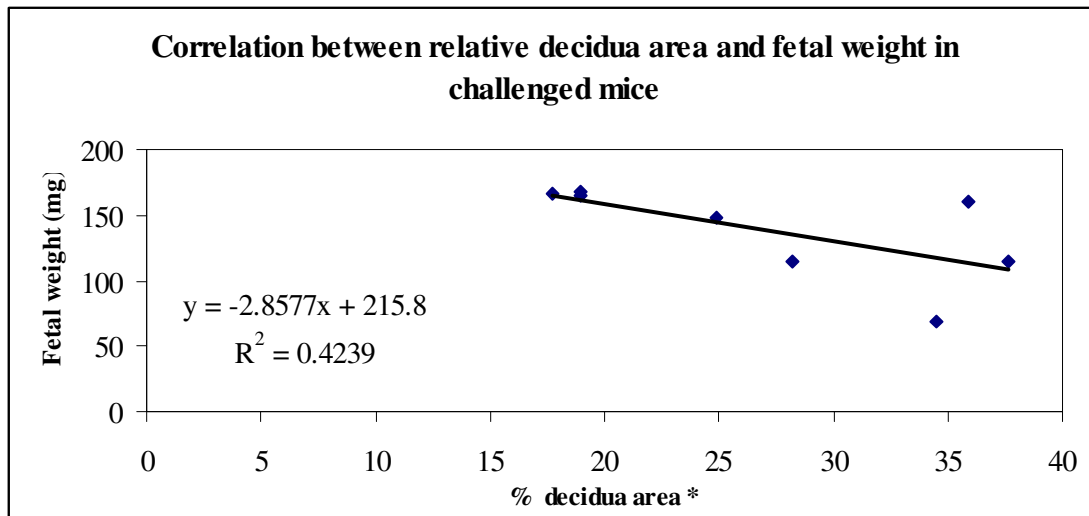
* Percentage of placental area occupied by the decidua layer

Figure 1.2. Hematoxylin and eosin staining of placental tissues from *C. rectus*-challenged mice and controls. Panel A depicts a control placenta (1×). Panel B depicts a placenta from the *C. rectus*-challenged group (1×). Notice the 3 placental layers separated by a yellow line: decidua (top), spongiotrophoblast (middle) and labyrinth (bottom). Panels C and D show the presence of inflammatory infiltrate in placentas from the *C. rectus*-challenge group at 20× magnification and at 40× magnification (E and F).
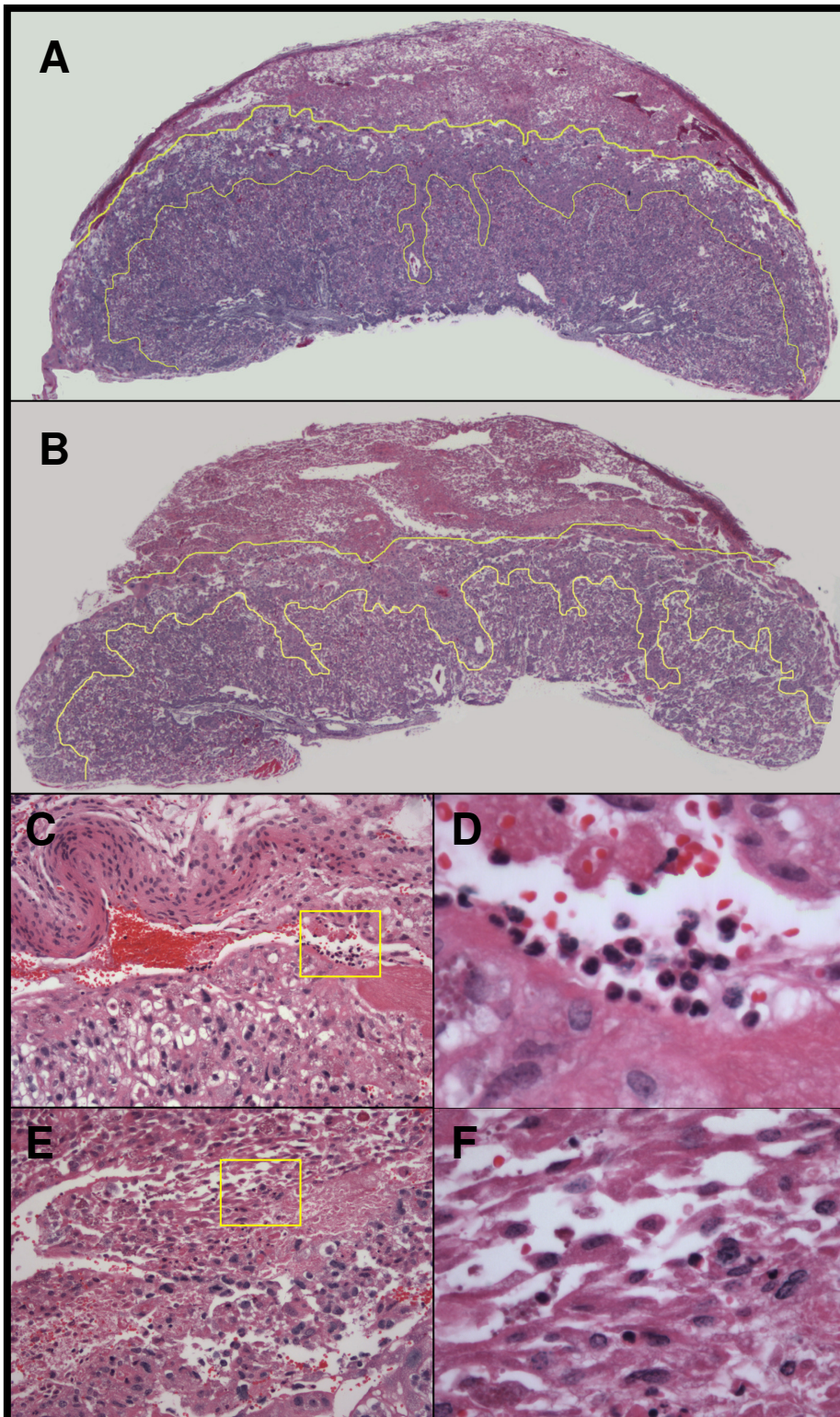
Figure 1.3. Detection of *Campylobater rectus* in the placenta of challenged mice. Panels A and B depict placentas of control mice. Panels C-F depict placentas of challenged mice in which *C. rectus* was detected. Red stain corresponds to F actin stained with Texas red-conjugated phalloidin. Green-yellow cells represent cells stained with a FITC-conjugated anti-*Campylobacter* antibody.
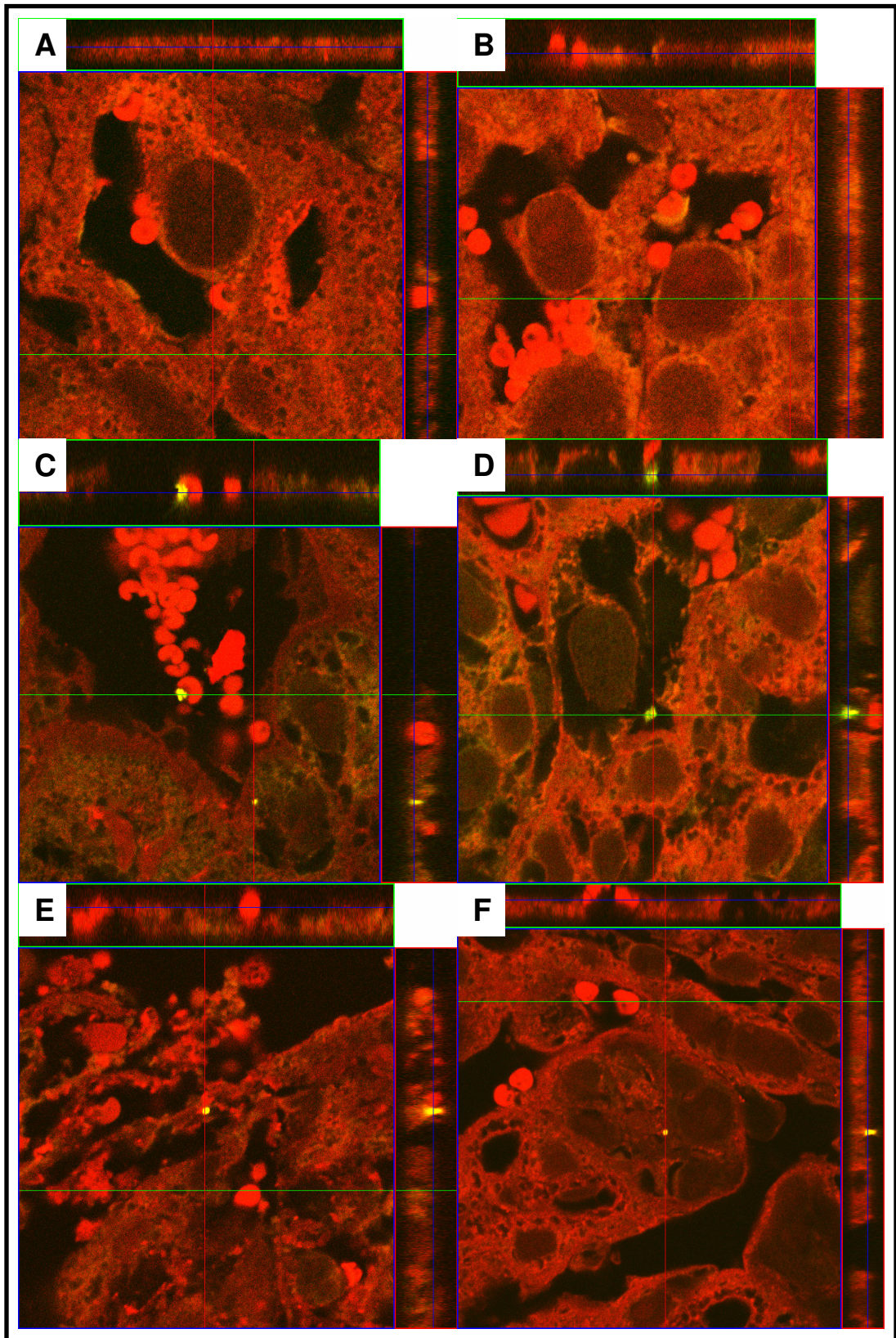
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Chapter 2

Original manuscript: *Campylobacter rectus* invades trophoblast, endothelial and epithelial cells and up-regulates *Vegf* expression in a murine trophoblast cell line.

Campylobacter rectus invades trophoblast, endothelial and epithelial cells and up-regulates *Vegf* expression in a murine trophoblast cell line.

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Abstract

Campylobacter rectus has been implicated as a possible cause in the association between periodontal disease and adverse pregnancy outcomes. A murine model has demonstrated that a distant *C. rectus* infection causes intra-uterine growth restriction and alterations in placental function. *C. rectus* has also been found to translocate to the fetal-placental unit and therefore, interact directly with placental tissues. **Aims:** to characterize the in vitro interactions of *C. rectus* with cells similar to those found in the placenta, ie. trophoblast and endothelial cells. **Methods:** Human endothelial (HUVEC), human trophoblast (BeWo), mouse trophoblast (SM9-1) and canine epithelial (MDCK) cell lines were grown and infected with *C. rectus* strains 314 (wild type), *C. rectus* S-layer deficient mutant or a *C. jejuni* strain (ATCC 43457) at a MOI of 100 and incubated at 37°C in 10% CO_2 for 5 h. Invasion was assessed by gentamicin protection assay and confocal microscopy after staining with a *Campylobacter*-specific antibody and actin-specific phalloidin (to mark individual cells). The expression of genes important for fetal/placental development in SM9-1 cells was also measured by real

time PCR. **Results:** *C. rectus* has the ability to invade human trophoblast and endothelial cells and canine epithelial cells. *C. rectus* was not demonstrated intracellularly in the mouse trophoblast cell line tested but was able to penetrate cell junctions. *C. rectus* infection of murine trophoblasts induced the up-regulation of the angiogenesis-related gene, *Vegf* which indicated the potential for murine trophoblasts to respond in vivo to the presence of the bacteria in placental tissues. In summary, *C. rectus* ability to invade epithelial, trophoblast and endothelial cell types or disrupt cell junctions might facilitate translocation of the bacteria from the periodontal sulcus to the fetal-placental unit. Once in the placental tissues, *C. rectus* could induce changes in trophoblast genes expression, such as *Vegf* up-regulation, that could result in an inflammatory response and alterations in placental morphology.

Introduction

Campylobacter rectus is a gram-negative, anaerobic and motile bacterium, with surface components, such as the flagellum, surface layer (S-layer), and cytotoxin, that have been reported as possible virulence factors (Okuda *et al.* 1997; Wang *et al.* 2000). This organism is part of the orange complex bacteria (Socransky *et al.* 1998) and is associated with the initiation and progression of periodontal disease (Ihara *et al.* 2003; Tanner *et al.* 1998). *C. rectus* has also been implicated in the association between periodontal disease and adverse pregnancy outcomes. Fetal exposure with *C. rectus* has been demonstrated to be higher in preterm than in full term neonates (Madianos *et al.* 2001). Moreover, the female sex hormones estradiol and progesterone significantly enhance the growth of *C. rectus* (Yokoyama *et al.* 2005), a finding that may relate with the ability of this organism to

disseminate systemically during pregnancy. Furthermore, other *Campylobacter* spp., *C. fetus* and *C. jejuni,* have been reported to be associated with abortion, premature labor and severe perinatal infection (Simor *et al.* 1986; Wong *et al.* 1990).

The effect of *C. rectus* on the fetal-placental unit has been studied using a murine model of intra-chamber challenge with *C. rectus* (Yeo *et al.* 2005). This model demonstrated that remote subcutaneous maternal infection increases fetal resorptions and fetal growth restriction. A further study also revealed that *C. rectus* challenge results in abnormal placental architecture (Offenbacher *et al.* 2005). Placentas from growth restricted fetuses of challenged mice showed decreased width of the vascular labyrinth and an increase in the decidual tissue and trophoblast layer, as compared to unchallenged dams. Challenged mice also showed areas of trophoblast invasion of the decidua, vasodilation of the fetal placental circulation in the junctional zone and focal areas of inflammatory infiltrate. Use of the same model has also demonstrated, by nested PCR and direct immunofluorescence, *C. rectus* translocation to the placenta of challenged mice (Bobetsis *et al.* 2007) (Diaz *et al.*, manuscript in preparation). While in the placenta, it is likely that *C. rectus* interacts with placental trophoblast cells that express pattern recognition receptors such as TLR-2 and TLR-4, inducing an inflammatory response to the presence of the bacteria (Abrahams *et al.* 2004; Guleria and Pollard 2000). Indeed, whole placental murine tissues have been demonstrated to show altered gene expression after a remote *C. rectus* infection (Diaz *et. al*. manuscript in preparation, Bobetsis *et al.* 2007). These changes could be the result of direct contact of the bacteria with placental tissues or part of a systemic inflammatory response. Analysis of whole placentas, however, has the limitation that it does not discriminate between the

different cell types present in the placenta, i.e. trophoblasts and endothelial cells; therefore, it is not clear whether *C. rectus* dysregulates trophoblast gene *C. rectus* may also have the ability to invade placental cells including endothelial cells and trophoblasts. Other *Campylobacter* species have been shown to readily invade host cells, a feature that may play a role in their virulence potential. *C. jejuni* invasion of enterocytes has been shown to induce oncotic changes on these cells with extensive cytoplasmic vacuolation and loss of plasma membrane integrity (Kalischuk *et al.* 2007). It has been claimed that invasion of bacteria into mammalian cells is an important mechanism to evade the immune system phagocytic cells and allows translocation of the bacteria and systemic dissemination (Li *et al.* 2008; Medina *et al.* 2003).

In this study we evaluated the ability of *C. rectus* to invade different cell lines including human endothelial and human and murine trophoblast cells. The effect of a *C. rectus* infection on murine trophoblasts was also investigated by looking at the levels of expression of 6 genes involved in placental development following a *C. rectus* challenge. These genes have all been shown to be expressed in mouse placental tissues and include *Vegfa*, *Pgf*, and their receptor *Flt-1*; the Vegfa-inducible anti-angiogenic gene *Vash1* and the vasoactive neuropeptide *Gal* and its receptor *Galr3* (Diaz *et al.* manuscript in preparation). In the mouse model, we have found that a distant infection with *C. rectus* abolished the relationship between patterns of expression of all these genes and fetal weight. Therefore, it is possible that direct bacterial interaction with placental cells, such as trophoblasts, dysregulates gene expression. The use of a cell line model to study these interactions would be advantageous to understand the mechanisms that regulate *C. rectus*-induced placental abnormalities.

Materials and Methods

Mammalian cell lines

Cells lines used for invasion experiments were the human trophoblast cell line BeWo (ATCC CCL-98), derived from a human choriocarcinoma (Pattillo and Gey 1968); the mouse trophoblast cell line SM9-1 (a kind gift from JS Hunt, University of Kansas Medical Center, Kansas City, KS) derived from a gestational day 9 Swiss-Webster mouse placenta (Bowen and Hunt 1999), the human endothelial cell line HUVEC (ATCC CRL-1730) and the canine epithelial cell line MDCK (ATCC CCL-34). Culture medium for BeWo cells was Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 10% fetal bovine serum (FBS), according to ATCC propagation instructions. Culture medium for SM9-1 cells was RPMI-1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and penicillin/streptomycin. Culture medium for HUVEC cells was F-12K supplemented with 0.1 mg/ml heparin, 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS) and 10% FBS. Culture medium for MDCK cells was Eagle's Minimum Essential Medium with 10% FBS according to ATCC propagation instructions. All cells were grown in T-25 flasks (Corning, Life Sciences, MA) or onto cover slips placed at the bottom of a well in 24-well plates for invasion assays. All cells were grown at 37°C in 10% CO_2.

Bacterial cultures

Campylobacter rectus strain 314, *Campylobacter rectus* 314 S-layer deficient mutant or *Campylobacter jejuni* strain ATCC 43457 were used for infection experiments. *C. rectus* strains were grown under anaerobic conditions at 37°C on Trypticase soy broth agar (Becton Dickinson and Company, Cockeysville, MD) supplemented with 0.2% yeast extract, 0.3% Phytone peptone, 0.2% NaCl, 0.3% ammonium formate, 0.4% sodium fumarate and 0.4% L-asparagine, adjusted to pH 7.8. *C. jejuni* was grown on Mueller Hinton agar (Oxoid) plates at 37°C under a microaerophilic atmosphere.

Invasion assays

Invasion assays were done according to a modification of the described gentamicin-protection protocol (Elsinghorst 1994). Bacterial strains were harvested from agar plates after 72 h incubation (48 h incubation yielded adequate growth for *C. jejuni*), diluted in tissue culture medium without antibiotics, and the concentration was determined by spectrophotometry at 600 nm.

Mammalian cell monolayers were washed 3 times with cell culture medium without antibiotics prior to inoculation with bacteria. Bacterial cells were added at a MOI of 100 to 80-100% confluent cells grown on 6 well-plates. After addition of the bacteria to the cells, plates were centrifuged at $250 \times g$ for 5 min, incubated for 2–24 h at 37°C in 10% CO_2 and washed with PBS. Tissue culture medium (1 ml) containing gentamicin (400 mg ml^{-1}) was added to half the wells, whereas medium with no antibiotics was added to the remaining

duplicate wells. The tissue culture plates were then incubated for another 2 h at 37°C and washed with PBS, followed by the addition to each well of 100 ml of 0.1% Triton X-100 in PBS and further incubation for 10–15 min at 37°C. Dilutions of each well were plated onto the appropriate agar, and colonies were enumerated after 5 days. Invasion efficiency was calculated as the percentage of colony-forming units (CFU) compared to the initial inoculum. Each invasion assay was performed in duplicate wells and repeated a minimum of three separate times. To evaluate the effect of medium or Triton X-100 on bacterial viability a suspension of bacterial cells was incubated with medium for 2 hours or with 0.1% Triton X-100 in PBS for 5 min at 37°C. CFUs were compared to those obtained from the initial non-treated inoculum.

Visualization of invasion by confocal microscopy

Cells were grown onto cover slips in 24 well plates to 80-100% confluency. Bacteria were added to cells as described above. After centrifugation of the plates, infection was allowed to proceed for 5 hours at 37°C in 10% CO_2. Infected cells and non-infected controls were then washed 3 times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After washing twice with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min. Blocking buffer (5% BSA, 1% goat serum and 0.2% Triton-X-100) was added for 1 h and cells were incubated overnight at 4°C with a FITC-conjugated anti-*Campylobacter* antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and Texas red-conjugated phalloidin (Invitrogen). After incubation with antibodies, cells were washed several times with PBS. Cover slips were then mounted on glass slides with 2 µl mounting

medium and observed using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope and 60X na 1.4 planapo and 100X na 1.3 plan neo fluar lenses. Random fields were captured using the LSM510 META software. Quantification of bacterial adhesion and invasion was done manually using the LSM510 META software and the ortho view to navigate the entire stack of images visualizing x, y and z planes. Student's t test was used to compare different groups.

Gene expression assays

SM9-1 cells were grown in 6 well plates and infected with *C. rectus* at a MOI of 100 for 6 hours as described above. After incubation with the bacteria, RNA was isolated following the Trizol protocol (Invitrogen). RNA pellets were dissolved in RNAse-free water and RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA quality was also checked by running samples in a Bioanalyser 2100 instrument (Agilent Technologies, Santa Clara, CA). Real-time PCR relative quantification was performed using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 1 µg RNA was reverse transcribed using the Omniscript RT kit (Qiagen, Valencia, CA), 10 µM random primers (Invitrogen) and 10 U RNase inhibitor (Ambion). Reactions were incubated at 37°C for 1 h. One µl of this reaction was added to a real time PCR reaction containing one of the following primers and probe assay mixtures: Vascular endothelial growth factor A (*Vegfa*) (Mm00437304_m1), FMS-like tyrosine kinase 1 (*Flt1*) (Mm00438980_m1), vasohibin 1 (*Vash1*) (Mm00616592_m1), galanin receptor 3 (*Galr3*) (Mm00443617_m1),

45

galanin (*Gal*) (Mm00439056_m1), placental growth factor (*Pgf*) (Mm00435613_m1). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (rodent *Gapdh* control reagents) and hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) (Mm01545399_m1) were used as housekeeping genes. The comparative C_T method was used to calculate the relative quantification according to Applied Biosystems ABI Prism 7700 Sequence Detection System User bulletin #2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Briefly, the ΔC_T was determined by subtracting the average C_T value of the housekeeping gene from the average C_T value of the target gene. Then the $\Delta\Delta C_T$ for each gene was calculated by subtracting the mean ΔC_T of the control from the ΔC_T of the infected cells.

Results

Quantification of invasion by gentamicin protection assay

Quantification of invasion of *C. rectus* to MDCK and BeWo cells by gentamicin protection assay did not detect any viable *C. rectus* invading the mammalian cell lines (Table 2.1). Quantification of the invasion of *C. jejuni*, which served as a positive control for the assay, showed bacterial invasion in both cell lines. Moreover, to assess the effect of cell growth medium or Triton X-100, used in the gentamicin protection assay, on *C. rectus*, the bacteria were incubated with either solution. Table 2.2 shows that the growth medium did not affect *C. rectus* viability while Triton X-100 killed 90% of the bacterial cells.

Quantification and visualization of adhesion and invasion by confocal microscopy

While the gentamicin protection assay did not show any *C. rectus* invasion to mammalian cells, direct visualization of bacterial cells by confocal microscopy showed that both *C. rectus* 314 and *C. rectus* S-layer mutant were present inside cells after 5 hours of infection (Figures 2.1-2.4). Therefore, bacterial adhesion and invasion were quantified in image stacks obtained by confocal microscopy. Bacteria were found attached to the outside of the mammalian cell, present in the cell junction within cells and co-localizing with actin, or inside the cells (cytoplasmic). As shown in Table 2.3, *C. jejuni*, *C. rectus* and *C. rectus* S-layer mutant adhered to SM9-1 murine trophoblast cells but invasion was negligible. In contrast, *C. jejuni*, *C. rectus* and *C. rectus* S-layer mutant invaded the human trophoblast cell line BeWo, the human endothelial cell HUVEC and the canine epithelial cell MDCK (p values < 0.05 when compared to SM9-1 cells). It was also observed that in most cases *C. jejuni* adhesion or invasion of cells was greater than that of *C. rectus*. Moreover, in most cases the adhesion and invasion of cell junctions by *C. rectus* S-layer mutant was greater than that of *C. rectus* wild-type strain.

Effect of C. rectus infection on gene expression in murine SM9-1 cells

No transcripts could be detected for the genes *Flt1, Vash1, Gal* and *Galr3*. *Vegf* and *Pgf* could be detected so their expression was measured at different time points. Table 2.4 shows the results from the real-time PCR assays. As seen in this table *Pgf* expression was not affected by the

presence of the bacteria but *Vegf* expression was increased 5-fold at the 2 hour infection time point.

Discussion

Host cell invasion is an important process in the pathogenesis of many bacteria and involves numerous steps: bacterial binding at specific receptor sites, signaling to the host cell, modification of intracellular host signal transduction pathways, membrane and cytoskeletal rearrangements, and eventual engulfment of the bacterium. The phenomenon of invasion of *Campylobacter* species to host cells has been studied mainly with the enteric pathogen *Campylobacter jejuni*. *C. jejuni* is a commensal inhabitant of the gastrointestinal tract of many animals and causes colitis and diarrhea in humans (Blaser *et al.* 1983). The invasiveness of *C. jejuni* in cultured epithelial cell lines has, in some cases, been correlated with colonization efficiency and disease outcome (Bacon *et al.* 2000; Fauchere *et al.* 1986; Hanel *et al.* 2004; Newell and Pearson 1984). The molecular mechanisms of *C. jejuni* invasion are still a matter of investigation. It has been shown that *C. jejuni* triggers membrane ruffling in the eukaryotic cell followed by invasion in a very specific manner first with its tip followed by the flagellar end (Krause-Gruszczynska *et al.* 2007). Some *C. jejuni* strains have also been shown to gain entry into epithelial cells by inducing rearrangements of the network of actin filaments that are concentrated just beneath the plasma membrane (Fauchere *et al.* 1986; Konkel and Joens 1989), while others trigger an unusual microtubule-dependent mechanism of uptake (Hu and Kopecko 1999). The invasiveness of *C. rectus* assessed by confocal microscopy, as reported in this study, appears to be similar to that

reported for other *Campylobacter* species (0.01 to 2 bacteria per cell), although the gentamicin resistance assay has shown that invasiveness varies considerably depending on the strain (Biswas *et al.* 2004; Friis *et al.* 2005). The number of internalized *Campylobacter* seems to increase with greater bacterial concentrations reaching a plateau at a MOI of 100-200, however, even with the most invasive strains only one to three bacteria are internalized per cell (Friis *et al.* 2005). The failure in the present study to detect viable *C. rectus* cells via the gentamicin protection assay may have been a consequence of the vulnerability of the bacteria to killing by the detergent used for cell lysis. However, it is also possible that *C. rectus* losses viability after entering the host cells as this microorganism differs from *C. jejuni* in that it requires an anaerobic environment for growth. During the invasion assay, the incubation conditions (90% air-10% CO_2) are only favorable to the epithelial cell and may be sufficient to cause lethal oxidative stress to *C. rectus.*

Microscopic observation of bacterial invasion showed that cells could cross the monolayer barrier by either a paracellular (through junctions between cells) or a transcellular (via the cell cytoplasm) route. Similar routes have been described for *C. jejuni* which has been seen to internalize into host cells and is detected in the cytoplasm (van Alphen *et al.* 2008) and also translocates trough cell junctions disrupting components of the tight junctions such as the protein occludin (Chen *et al.* 2006). Either bacterial translocation via a paracellular route or internalization could be useful for *C. rectus* ability to cross the oral epithelial barrier, and then interact with endothelial cells to enter the circulation and migrate to distant sites such as the fetal-placental unit.

The role of the S-layer in *Campylobacter* virulence remains unclear. However, the only two *Campylobacter* species generally associated with adverse pregnancy outcomes, *C. rectus* and *C. fetus*, are those in which an S-layer has been described (Thompson 2002). It has been speculated that the S-layer could act a mechanism of evasion of the immune system as it confers *C. rectus* and *C. fetus* the ability to prevent complement binding to the cells thus avoiding complement-dependent lysis (Okuda *et al.* 1997). The absence of an S-layer in the present study resulted, in some cases, in increased adhesion and internalization of *C. rectus*. Similar results have been previously observed by Borinski and Holt (1990) who reported that spontaneous S-layer deficient mutants bind better to human gingival fibroblasts than strains with an S-layer. On the contrary Wang *et al.* (2000) reported that *C. rectus* isogenic mutant strains lacking the S-layer protein gene (crsA) were 30 to 50% less adherent to HEp-2 epithelial cells than the wild type. Wang *et al.* (2000) included the spontaneous mutants in their study and found similar results with the isogenic mutant (the S-layer spontaneous mutant was less adherent than the wild type) therefore they attribute the differences in results with Borinski and Holt (1990) to the type of mammalian cell used. It is evident from this study that differences in adhesion and invasion are seen depending on the mammalian cell line tested. It is also evident from the results obtained by this study that increased adhesion does not always reflect increased internalization of the bacteria into the host cell.

We also observed in this study that cytoplasmic internalization of *C. rectus* was a rare event in murine trophoblasts while the presence of cytoplasmic bacteria was commonly seen in human trophoblasts. Differences in cell surface characteristics between the human and mouse trophoblast cells are most likely responsible for the lack of cytoplasmic invasion in the

mouse. It is known that differences exist in the response of mouse and human epithelial cells to other *Campylobacter* species such as *C. jejuni*. Indeed, Rinella *et al.* (2006) have demonstrated that only human colonic epithelia exposed to *C. jejuni* show altered gene expression, while the murine colonic cells remained unresponsive. Ruiz-Palacios *et al.* (2003) have also observed that *C. jejuni*, which normally does not bind to Chinese hamster ovary cells, binds avidly when the cells are transfected with a human alpha1,2-fucosyltransferase gene thus demonstrating the lack of a *Campylobacter* receptor in the murine cells. Although the murine model has proven useful in studying the role of *C. rectus* in adverse pregnancy outcomes it is possible that *Campylobacter* spp. are human-specific pathogens and therefore, the murine model underestimates the potential damage caused by *C. rectus* to the fetal-placental unit.

Previous experiments have demonstrated that placental tissues from BALC/c mice express the angiogenesis-related genes *Vegf* and *Pgf*. VEGF-A is the major player in angiogenesis among the VEGF family and activates two tyrosine-kinase receptors, VEGFR1 (FLT1) and VEGFR2 (KDR in humans/Flk-1 in mice) (Shibuya 2006). PGF is also a member of the VEGF family, with significant amino-acid homology to VEGF (Iyer *et al.* 2001) and shown to be expressed at high levels in human trophoblast cells and umbilical vein endothelial cells (Kleine *et al.* 2001). Here we demonstrate that a murine-derived trophoblast cell line, SM9-1, expresses these two genes and that *C. rectus* upregulates *Vegf* expression after 2 hours of infection. Up-regulation of *Vegf* after a bacterial challenge has been demonstrated to occur in human intestinal epithelial cells challenged with an adherent pro-inflammatory *Escherichia coli* strain (Cane *et al.* 2007). The mechanism by which VEGF was up-regulated involved the

interaction of a bacterial adhesin with a cell surface molecule, the brush border-associated decay accelerating factor (DAF, CD55) that acts as a bacterial receptor, and promotes the activation of a Src protein kinase upstream of the activation of Erk and Akt signaling pathways. Up-regulation of VEGF by bacteria has also been documented for other gastrointestinal pathogens including *Helicobacter pylori* (Kitadai *et al.* 2003; Strowski *et al.* 2004) and *Listeria monocytogenes* (Sato *et al.* 2007) which increased the expression of VEGF mRNAs through a Erk-mediated pathway. It is also possible that *C. rectus* activates the production of pro-inflammatory mediators in murine trophoblasts cells which, in turn, could up-regulate *Vegf*. Although the regulatory mechanisms of VEGF expression by inflammatory mediators are not completely understood evidence, exists that cytokines such as IL-1ß, TNF-α and IL-6 up-regulate VEGF (Angelo and Kurzrock 2007). As the *Vegf* receptor Flt-1 was not detected in murine trophoblasts, we can assume that if *Vegf* up-regulation happens in vivo in placental trophoblast cells, its effect is probably directed to the adjacent endothelial cells, increasing vascularity, which in turn will increase nutrient and oxygen supply and facilitate an inflammatory response.

In summary, we have demonstrated that *C. rectus*, a periodontal pathogen implicated in the relationship between periodontal disease and adverse pregnancy outcomes is capable of invasion of epithelial, endothelial and trophoblast cell lines, a characteristic that might allow the bacteria to translocate from a distant site of infection to the fetal placental unit. *C. rectus* interaction with human and murine trophoblasts was also distinct indicating that host characteristics may affect *C. rectus* invasive ability. Moreover, *C. rectus* challenge of a murine trophoblast cell line induced an up-regulation of the important angiogenesis-related

gene, *Vegf*, which may indicate the potential for murine trophoblasts to respond in vivo to the

presence of the bacteria in placental tissues.

Table 2.1. Invasion of *Campylobacter rectus*, *C. rectus* S-layer mutant and *C. jejuni* into BeWo and MDCK cells as measured by the gentamicin protection assay[1].

	C. rectus	*C. rectus* S-layer mutant	*C. jejuni*
BeWo	ND	ND	0.011%±0.005
MDCK	ND	ND	0.023%±0.003

[1] Invasion efficiency was calculated as the percentage of colony-forming units (CFU) compared to the initial inoculum.

Table 2.2. Effect of MDCK cell growth medium and 0.1% Triton X-100 on *Campylobacter*

rectus viability

Initial inocolum	MDCK growth medium-treated bacterial cells	Triton X-100-treated bacterial cells
$3.43 \pm 0.8 \times 10^5$	$3.84 \pm 0.4 \times 10^5$	$2.00 \pm 1.2 \times 10^4$

Bacterial cells were incubated with growth medium for 2 h at 37ºC and with Triton X-100 for

5 min at 37ºC

Table 2.3. Adhesion and invasion of *C. rectus* and a *C. rectus* S-layer mutant to different cell lines. *C. jejuni* 43457 served as a positive control. *p<0.05. **p<0.001.

Cell line	Bacteria	# bacteria adhered/cell	# bacteria co-localizing with actin (in cell junctions)/cell	# invasive bacteria /cell (cytoplasmic)
SM9-1	*C. rectus* 314	0.50±0.41	0.44±0.10	0.00±0.00
	C. rectus 314 S-layer mutant	1.74±1.04 *	1.16±0.67 *	0.04±0.03
	C. jejuni 43457	4.79±2.82	1.53±0.71	0.00±0.00
BEWO	*C. rectus* 314	0.43±0.52	0.08±0.11 *	5.51±3.18 *
	C. rectus 314 S-layer mutant	0.54±0.54 * *	0.46±0.29 ** **	0.75±0.29 *
	C. jejuni 43457	5.30±1.75	2.83±0.32	2.96±0.77
HUVEC	*C. rectus* 314	0.49±0.23 **	0.40±0.23 **	0.11±0.05
	C. rectus 314 S-layer mutant	2.17±0.83 *	1.40±0.38 * *	0.26±0.14 *
	C. jejuni 43457	0.92±0.32	0.83±0.14	0.55±0.26
MDCK	*C. rectus* 314	0.26±0.36	0.10±0.12 *	0.69±0.25
	C. rectus 314 S-layer mutant	0.35±0.26 * *	0.00±0.00 * *	1.84±1.56 *
	C. jejuni 43457	3.83±2.40	1.40±0.65	1.84±1.19

Table 2.4. Effect of *C. rectus* infection on gene expression in murine SM9-1 cells

Gene	Infection time	mRNA ratio challenged/controls
Vegf	2 h	5.2±1.8
	6 h	1.5±0.9
	12 h	1.7±0.8
Pgf	2 h	1.6±1.9
	6 h	0.8±1.0
	12 h	1.3±0.9

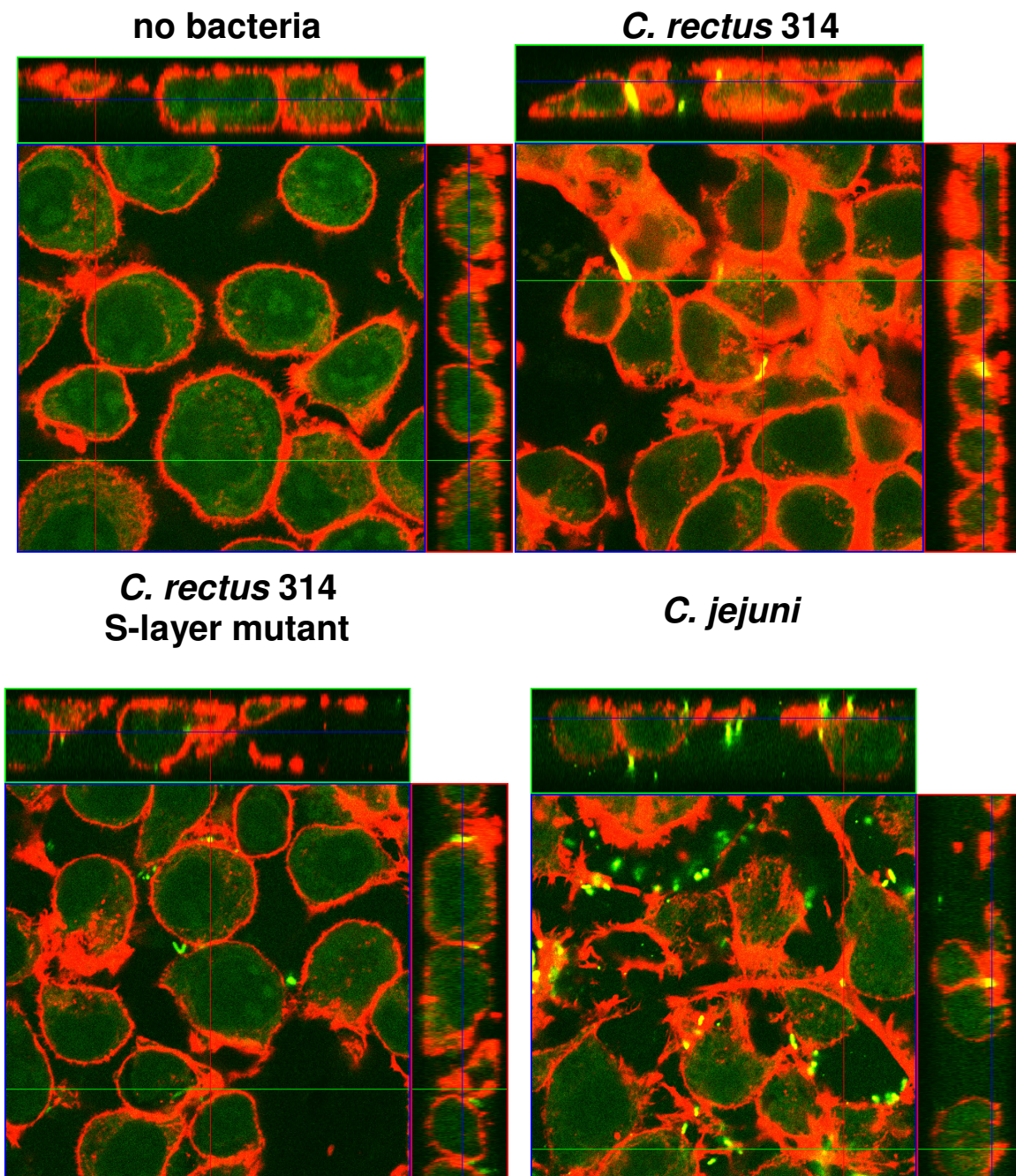Figure 2.1. Adhesion and invasion of SM9-1 cells with *C. rectus*, *C. rectus* S-layer mutant and *C. jejuni*

no bacteria

C. rectus 314

C. rectus 314 S-layer mutant

C. jejuni

Figure 2.2. Adhesion and invasion of BeWo cells with *C. rectus*, *C. rectus* S-layer mutant and *C.*

jejuni

no bacteria

C. rectus 314

**C. rectus 314
S-layer mutant**

C. jejuni

Figure 2.3. Adhesion and invasion of HUVEC cells with *C. rectus*, *C. rectus* S-layer mutant and
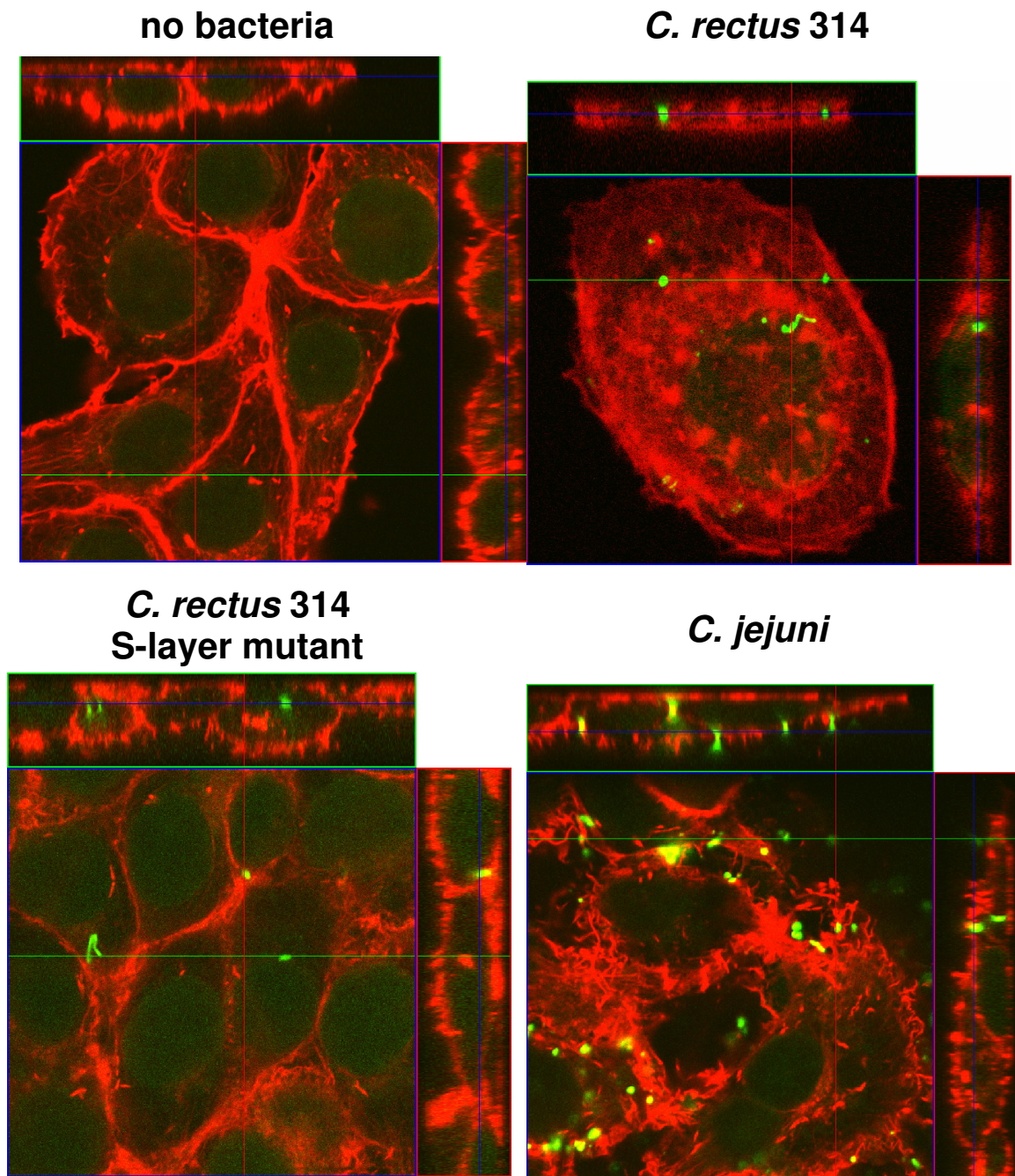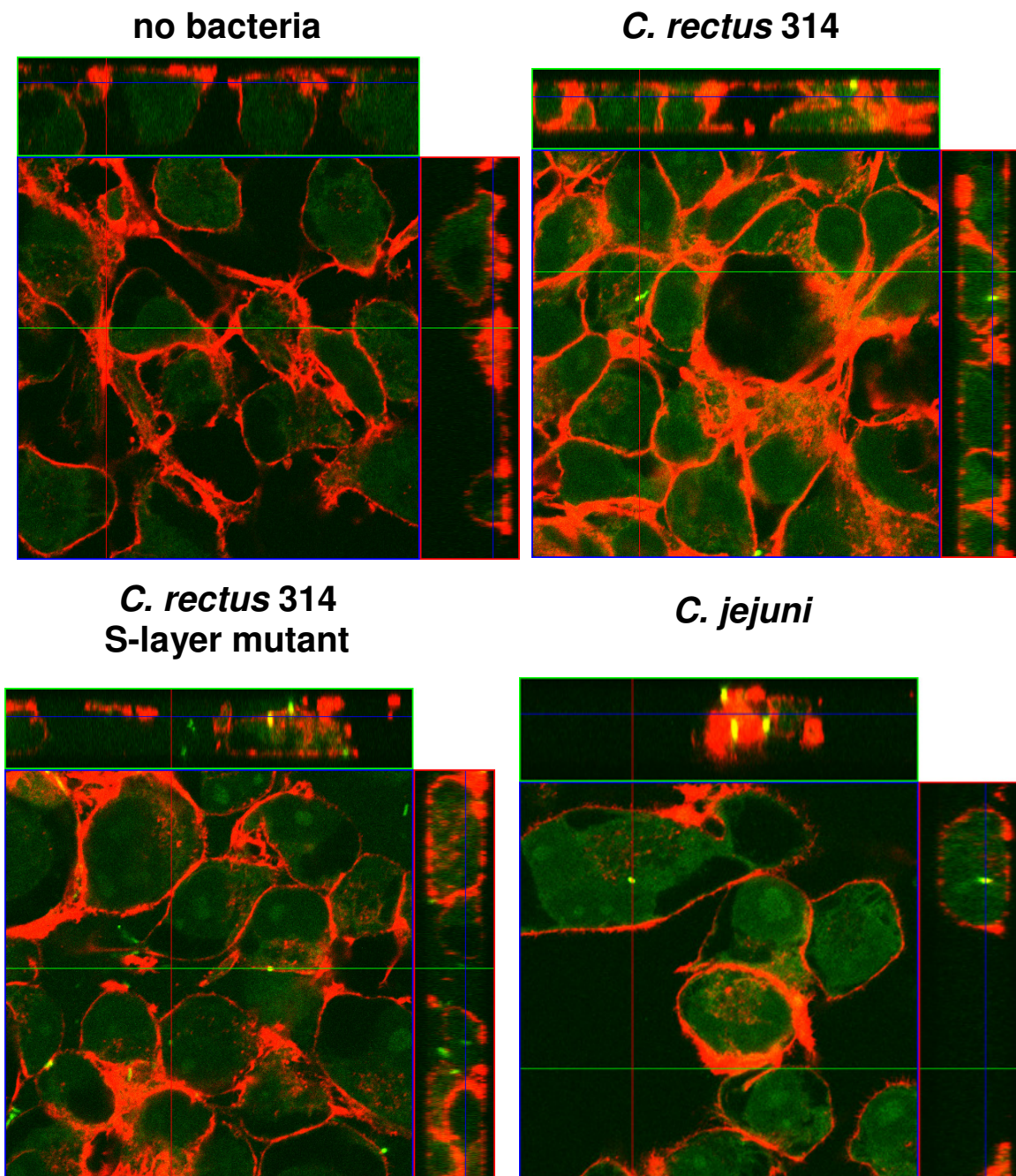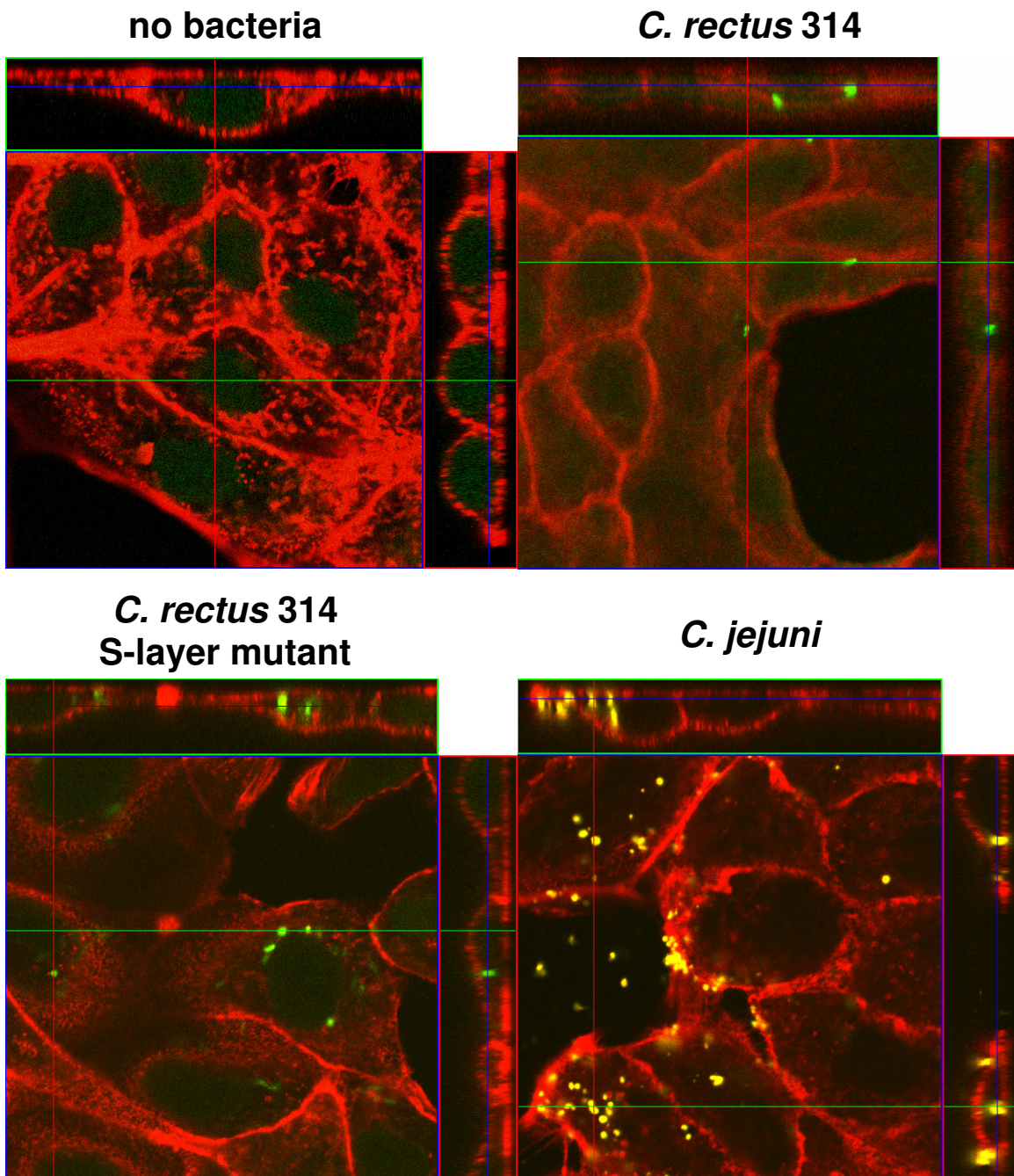
C. jejuni

no bacteria

C. rectus 314

C. rectus 314
S-layer mutant

C. jejuni

Figure 2.4. Adhesion and invasion of MDCK cells with *C. rectus*, *C. rectus* S-layer mutant and

C. jejuni

no bacteria

***C. rectus* 314**

***C. rectus* 314**
S-layer mutant

C. jejuni

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