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Hepatitis A virus cellular receptor 2 (HAVCR2) is decreased with viral infection and regulates pro-labour mediators

Running title: HAVCR2 and viral infection in pregnancy

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ABSTRACT

PROBLEM: Intrauterine infection caused by viral infection has been implicated to contribute to preterm birth. Hepatitis A virus cellular receptor 2 (HAVCR2) regulates inflammation in non-gestational tissues in response to viral infection.

METHOD OF STUDY: The aims of this study were to determine the effect of: (i) viral dsRNA analogue polyinosinic:polycytidylic acid (poly(I:C)) on HAVCR2 expression; and (ii) *HAVCR2* silencing by siRNA (siHAVCR2) in primary amnion and myometrial cells on poly(I:C)-induced inflammation.

RESULTS: In human fetal membranes and myometrium, *HAVCR2* mRNA and protein expression was decreased when exposed to poly(I:C). Treatment of primary amnion and myometrial cells with poly(I:C) significantly increased the expression and release of pro-inflammatory cytokines *TNF*, *IL1A, IL1B* and *IL6*; the expression of chemokines *CXCL8* and *CCL2*; the expression and secretion of adhesion molecules *ICAM1* and *VCAM1*; and *PTGS2* and *PTGFR* mRNA expression and the release of prostaglandin $PGF_{2\alpha}$. This increase was significantly augmented in cells transfected with siHAVCR2. Furthermore, mRNA expression of anti-inflammatory cytokines *IL4* and *IL10* was significantly decreased.

CONCLUSIONS: Collectively, our data suggests that HAVCR2 regulates cytokines, chemokines, prostaglandins and cell adhesion molecules in the presence of viral infection. This suggests a potential for HAVCR2 activators as therapeutics for the management of preterm birth associated with viral infections.

Keywords: HAVCR2; viral infection; inflammation; fetal membranes; myometrium

INTRODUCTION

Preterm birth complications are responsible for over 1 million annual neonatal deaths [1], and for survivors, there are lifelong effects such as impaired neurodevelopmental functioning and increased risk of developing chronic disease in adulthood [2]. Preterm labour is a syndrome as determined by increased uterine contractility, cervical ripening and/or fetal membrane rupture, processes which may be pathologically activated [3]. While the underlying aetiologies of preterm labour remain poorly understood, numerous studies suggest that intrauterine infection is a critical mechanism, and may account for 40% of preterm births [4]. However, this number may be underestimated, as many

infections are subclinical and remain undetected [5]. In a healthy pregnancy, the cervix protects the uterus and growing fetus from ascending bacterial infections [6]. Viral infection of the cervix, however, compromise the innate immune response, predisposing to ascending intrauterine infection [7]; changes in the expression and function of toll-like receptors (TLRs) and antimicrobial products causes activation of the terminal effector pathways that lead to preterm birth.

The HAVCR (Hepatitis A virus cellular receptor) family of genes has garnered much attention due to its association with multiple diseases, including allergy, asthma and autoimmunity [8]. In humans, HAVCR2 is expressed on T helper 1 (Th1) cells, and plays critical roles in regulating immune cell activity, especially in response to viral infection [9]. Studies in non-gestational tissues have indicated HAVCR2 to serve opposing roles in the innate and adaptive immune response [10]. It has been proposed that HAVCR2 expressed by innate immune cells interacts with TLRs to promote inflammation during the early phase of the immune response. As the immune response progresses, Th1 cells are generated. During the later stages of the immune response, HAVCR2 expression on Th1 cells becomes upregulated and this T cell driven inflammation is then terminated [10]. Specifically, HAVCR2 activation has been shown to reduce T cell-mediated cytotoxicity [11] and decrease interferon (IFNG) production [12]. More recently, experimental animal models of inflammatory diseases, including autoimmune arthritis and encephalitis, have implicated HAVCR2 to predominately exert anti-inflammatory effects *in vivo* [12].

There is a growing body of evidence that HAVCR2 exerts anti-inflammatory actions in response to viral infection. For example, enhanced HAVCR2 activity in a murine model of influenza infection reduces the risk of morbidity and mortality post-infection [13]. Moreover, HAVCR2 signalling inhibits T-cell mediated cytotoxity activity in response to T-lymphotropic Virus Type I infection [10]. Other studies have shown that HAVCR2 blockade in human $CD14^+$ monocytes results in a significant increase in the production of the pro-inflammatory cytokine IL12, as well as a significant decrease in immunoinhibitory receptor PDCD1expression [14]. These studies suggest HAVCR2 has an anti-inflammatory role during viral infection.

There have been recent studies showing that HAVCR2 may be important in the maintenance of a healthy pregnancy. For example, pregnant women with HAVCR2 positive NK and $CD8^+$ T cells in their peripheral blood are associated with a significant down-regulation of pro-inflammatory cytokine production when compared to pregnant women without HAVCR2 expressed NK and $CD8^+$ T cells [15]. HAVCR2 may also be involved in regulating inflammation in women with early onset preeclampsia. Women with preeclampsia had decreased HAVCR2 expression by cytotoxic T

cells and CD56^{dim} NK cells when compared to healthy pregnant women [16]. Furthermore, cytotoxic T and CD56^{dim} NK with diminished HAVCR2 expression was associated with increased cytotoxic activity in preeclamptic women. These findings suggest that loss of *HAVCR2* may enhance the systemic inflammatory response that is associated with the pathophysiology of preeclampsia.

To our knowledge, the expression or role of HAVCR2 has not been investigated in human fetal membranes or myometrium. Given the emerging role of viral infection leading to preterm birth [4, 17, 18], and the role of HAVCR2 during the immune response to viral infections [9], the aims of this study were (i) to determine the expression of HAVCR2 in fetal membranes and myometrium treated with the viral dsRNA analogue poly(I:C); and (ii) to determine the effect of *HAVCR2* silencing by siRNA in primary myometrial and amnion cells on poly(I:C)-stimulated pro-inflammatory and pro-labour mediators.

METHODS

Tissue collection

The Research Ethics Committee of Mercy Hospital for Women approved this study. Written, informed consent was obtained from all participating women. All tissues were obtained from women who delivered healthy, singleton infants. Myometrial biopsies were taken at the same time that the placenta was collected (for fetal membranes) at the time of Caesarean section delivery in the absence if labour. Myometrial biopsies were obtained from the upper margin of the lower uterine segment incision during the Caesarean section. All tissues were brought to the research laboratory and processed within 15 min of the Caesarean delivery. Women in the study were aged between 18-40 years old and non-obese (BMI <30). The racial population for all women were mixed. ~~All women had normal placental pathology, and Caesarean section delivery was performed on uncomplicated pregnancies at term gestation.~~ Indication for Caesarean section delivery was due to breech presentation or previous Caesarean section. For immunohistochemistry studies, fetal membranes and myometrial tissue samples were obtained from three preterm cases (gestational age 29-34 weeks) and three term cases (gestational age 37-40 weeks), all in the absence of labour. Indications for preterm delivery (in the absence of labour) were placenta praevia or placental abruption. For all term and preterm samples, women with any underlying medical conditions such as diabetes, asthma, polycystic ovarian syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with multiple pregnancies, obese women, fetuses with chromosomal abnormalities were excluded. In addition, all cases were hepatitis serology negative and did not have any symptoms of viral illness at the time of sample collection.

Immunohistochemistry (IHC)

IHC was performed on paraffin sections as described previously [19] using the IHC Select® HRP Detection Set (Merck Millipore; Billerica, MA, US). Briefly, sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM Tris, 1 mM EDTA, pH 9.0 for 10 min followed by 20 min incubation) and then endogenous peroxidases were inactivated by adding 3% hydrogen peroxide for 10 min. After blocking (Blocking Reagent: normal goat serum in PBS) for 5 mins, sections were incubated with 1 μg/ml rabbit polyclonal anti-HAVCR2 (SAB2103930; Sigma-Aldrich; St. Louis, MO, USA) in 1% (wt/vol) bovine serum albumin in PBS and incubated in a humidity chamber at 4°C overnight. Binding sites were labelled with biotin conjugated rabbit anti-goat IgG antibody followed by the streptavidin-HRP. Haematoxylin was used for nuclear-staining. Positive controls, which were either human placenta or composite slides with tonsil, spleen and breast tumour, were included in each run. Negative control slides, where primary antibody was replaced with rabbit IgG, were also performed.

Tissue explant culture

Tissue explants were performed to determine the effect of viral infection on the expression of HAVCR2 in fetal membranes and myometrium. For these studies, fetal membranes and myometrial biopsies were obtained at the same time from women undergoing elective Caesarean section in the absence of labour who delivered healthy, singleton infants at term (37-40 weeks gestation). Tissue explants were performed as previously described on fetal membranes obtained 2 cm from the peri-placental edge from non-labouring women at the time of term Caesarean section [20]. Briefly, fresh fetal membranes were placed in DMEM at 37^{o}C in a humidified atmosphere of 8% O_2 and 5% CO_2 for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg wet weight/well). Myometrium was incubated in a humidified atmosphere of 21% O_2 and 5% CO_2, in 24-well tissue culture plates (50 mg wet weight/well). Tissues were incubated in 50 μg/ml poly(I:C) (Sigma-Aldrich; St. Louis, MO, USA) for 20 h. The concentration of poly(I:C) is based on previous studies in human gestational tissues ~~fetal membranes~~[21, 22]. After final incubation, tissue was collected and stored at -80^{o}C for analysis by Western blot as detailed below. Experiments were performed on fetal membranes and myometrium obtained from five patients.

Gene silencing of *HAVCR2* with siRNA

Fresh amnion (obtained 2 cm from the peri-placental edge) and myometrium were obtained from women who delivered healthy, singleton infants at term (37-41 weeks gestation) undergoing elective Caesarean section in the absence of labour. Primary amnion and myometrial cells were

isolated and cultured as we have previously described [23]. Cells at approximately 50% confluence were transfected using Lipofectamine 3000 according to manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia). HAVCR2 siRNA (siHAVCR2) and negative control (siCONT) was obtained from Origene (Rockville, MD, USA). Cells were transfected with 200 nM siHAVCR2 or 200 nM siCONT in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA for myometrial cells, or 2% FBS for amnion cells) with or without 5 μg/ml poly(I:C), and the cells were incubated at 37°C for an additional 24 h. A lower concentration of poly(I:C) was used for cells (5 μg/ml) compared to tissue explants (50 μg/ml) as this concertation is sufficient to elicit an inflammatory response without any adverse effects on cell toxicity [24]. Cells were collected and stored at −80°C until assayed for mRNA expression by qRT-PCR and protein expression by Western blotting as detailed below. Media was collected and stored at −80°C until assayed for cytokine release as detailed below. Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay as we have previously described [23]. As previously reported, the response to poly(I:C) between patients varied greatly [19]. Thus, data is presented as fold change in expression relative to the expression level in the poly(I:C)-stimulated siCONT transfected cells, which was set at 1. Data could not be normalised to siCONT transfected cells alone as some of the readings were 0. Each experiment was performed from amnion and myometrium obtained from five patients.

RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA extractions and qRT-PCR was performed as previously described [19]. RNA quality and integrity were measured using a NanoDrop ND1000 and determined via the A_{260}/A_{280} ratio. RNA (0.5 μg for tissues and 0.2 μg for cells) was converted to cDNA using the Tetro cDNA synthesis kit (Bioline; Alexandria, NSW, Australia) according to the manufacturer's instructions. The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia) using 100 nM of pre-designed and validated QuantiTect primers (Qiagen; Chadstone Centre, Vic, Australia). The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. A positive cDNA sample, from human placenta known to express HAVCR2 [25], was included to confirm primer specificity. Average gene Ct values were normalised against two housekeeping genes (β2-Microglobulin (*B2M*) and succinate dehydrogenase complex subunit A (*SDHA*)). Of note, there was no effect of experimental treatment on *B2M* or *SDHA* mRNA expression. Fold differences were determined using the comparative Ct method.

Western blotting

Western blotting was performed as previously described [19]. Membranes were incubated in 1 μg/ml rabbit polyclonal anti-HAVCR2 (SAB2103930; Sigma-Aldrich; St. Louis, MO, USA). Semi-quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories, Hercules, CA, USA). The levels of HAVCR2 were normalised to the levels of ACTB (Sigma, St. Louis, MO, USA).

Enzyme immunoassays

Assessment of cytokine and chemokine release of IL6 and CXCL8was performed using the CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies; Mulgrave, Vic, Australia). The release of sICAM1 and sVCAM1 was performed by sandwich ELISA from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. The release of $PGF_{2\alpha}$ into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, NSW, Australia). The interassay and intraassay coefficients of variation for all assays were less than 10%.

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For two sample comparisons, either a paired or unpaired Student's t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons, the homogeneity of data was assessed by the Bartlett's test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (with LSD post-hoc testing to discriminate among the means). Statistical significance was ascribed to a P value <0.05. Data were expressed as mean ± SEM.

RESULTS

Localisation of HAVCR2 in term and preterm fetal membranes and myometrium

The first aim of this study was to determine the localisation of HAVCR2 in human fetal membranes and myometrium. For these IHC studies, samples were obtained from women at the time of term and preterm Caesarean section in the absence of labour. Fetal membranes are demonstrated in Figure 1A, with representative images from 1 patient for both preterm and term gestations. Preterm and term myometrium is depicted in Figure 1B. In fetal membranes, at both preterm and term gestations, HAVCR2 was present in amnion epithelium, chorionic trophoblasts, decidua, and in the

fibroblasts of the connective tissue layer (Figure 1A). In myometrium, HAVCR2 staining was present in the longitudinal and transverse muscle fibres (Figure 1B). No non-specific staining was present in the negative controls for fetal membranes (Figure 1A) and myometrium (Figure 1B).

Effect of viral infection on fetal membranes and myometrium on HAVCR2 expression

To determine the effect of viral infection on HAVCR2 expression in fetal membranes and myometrium, tissues were treated with the viral dsRNA analogue poly(I:C). Figure 2 demonstrates that HAVCR2 mRNA and protein expression is significantly decreased in fetal membranes (Figures 2A,B) and myometrium (Figures 2C,D) treated with poly(I:C).

Effect of siHAVCR2 on the expression and secretion of pro-inflammatory cytokines and chemokines

The efficacy of siHAVCR2 is demonstrated in Supplementary Figure 1. In amnion cells, there was a 55% decrease in *HAVCR2* mRNA expression (Supplementary Figure 1A). In myometrial cells, there was a 45% decrease in *HAVCR2* mRNA expression (Supplementary Figure 1C). A MTT cell viability assay showed no difference in absorbance between cells transfected with siCONT or siHAVCR2 in amnion (Supplementary Figure 1B) or myometrium (Supplementary Figure 1D).

We next sought to determine the effect of siHAVCR2 on poly(I:C)-induced expression and secretion of pro-inflammatory cytokines and chemokines in primary cells isolated from fresh amnion (Figure 3) and myometrium (Figure 4). In both amnion and myometrial siCONT transfected cells, treatment with the viral analogue poly(I:C) significantly increased *IL1A*, *IL1B*, *IL6*, *CXCL8* and *CCL2* mRNA expression and release of IL6 and CXCL8. Poly(I:C) treatment in amnion cells significantly increased *TNF* mRNA expression, however it was not increased in myometrial cells. The effect of siHAVCR2 in amnion and myometrial cells was a significant increase in the expression of poly(I:C)-stimulated *TNF*, *IL1A*, *IL1B*, *IL6*, *CXCL8* and *CCL2* mRNA expression and release of IL6 and CXCL8.

Effect of siHAVCR2 on the expression of anti-inflammatory cytokines

The effect of siHAVCR2 on poly(I:C)-induced expression of anti-inflammatory cytokines in primary cells isolated from fresh amnion (Figure 5A,B) and myometrium (Figure 5C,D) was also determined. In siCONT transfected amnion and myometrial cells, there was no change in *IL4* or *IL10* mRNA expression with poly(I:C) treatment. However, in *siHAVCR2* transfected amnion and myometrial cells, the presence of poly(I:C) resulted in a significant reduction in both *IL4* and *IL10* mRNA expression. Of note, under basal conditions there was no significant effect in *IL4* or *IL10*

mRNA expression in siHAVCR2 transfected amnion and myometrial cells when compared to siCONT transfected cells (Supplementary Figure 2).

Effect of siHAVCR2 on the prostaglandin pathway

Treatment with poly(I:C) in siCONT transfected cells significantly increased both *PTGS2* and *PTGFR* mRNA expression in amnion cells (Figures 6A,B) and myometrial cells (Figures 6D,E). In siHAVCR2 transfected cells, there was a significant increase in poly(I:C)-induced *PTGS2* and *PTGFR* mRNA expression in both amnion and myometrial cells. There was a significant increase in the release of $PGF_{2\alpha}$ in the media from siCONT transfected cells treated with poly(I:C) in both amnion and myometrial cells (Figures 6C,F). This effect was significantly augmented in siHAVCR2 transfected cells.

Effect of siHAVCR2 on the expression and secretion of adhesion molecules

The effect of siHAVCR2 on poly(I:C)-induced expression and secretion of adhesion molecules in primary cells isolated from fresh amnion and myometrium is depicted in Figure 7. In siCONT transfected cells, there was a significant increase in *ICAM1* and *VCAM1* mRNA expression (Figures 7A,C,E,G) and release (Figures 7B,D,F,H) with poly(I:C) treatment. This effect was significantly augmented in siHAVCR2 transfected cells, however the increase in sVCAM1 concentration in siHAVCR2 transfected myometrial cells (Figure 7H) did not reach statistical significance.

DISCUSSION

An association between viral infection and preterm labour has been reported by a number of epidemiologic studies [17, 26]. Our study demonstrates that fetal membranes and myometrium treated with the viral dsRNA analogue poly(I:C), a known inducer of pro-inflammatory mediators in human gestational tissues [19, 23], decreases *HAVCR2* mRNA and protein expression. In primary cells isolated from amnion and myometrium, *HAVCR2* silencing by siRNA is associated with an increase in pro-inflammatory cytokines and chemokines stimulated by poly(I:C). *HAVCR2* silencing by siRNA also increased *PTGS2*, *PTGFR*, *ICAM1* and *VCAM1* mRNA expression, and the production of prostaglandins and cell adhesion molecules when in the presence of poly(I:C). There was also a congruent decrease in poly(I:C)-induced expression of anti-inflammatory cytokines *IL4* and *IL10* in HAVCR2 siRNA transfected cells. Taken together, our studies suggest a functional role for HAVCR2 in down-regulating the inflammatory response in the presence of viral infection.

HAVCR2 contains multiple target sites for O- and N-linked glycosylation [12, 27, 28]. In this study, the highly glycosylated form of HAVCR2 (~50 kDa) was detected in fetal membranes and myometrium. In contrast, the un-glycosylated form of HAVCR2 (33 kDa) was not detected in these tissues. Notably, glycosylation of HAVCR2 plays an important role in the regulation of immune responses [12, 29].

Studies in non-gestational tissues have reported HAVCR2 expression to be decreased during human T-lymphotropic virus type I infection [29]. HAVCR2 expression on cytotoxic T cells is significantly reduced in patients with acute resolving hepatitis C infection compared to individuals with chronic infections [30]. Further, HAVCR2 expression in intratumoral DCs are downregulated in mice stimulated with poly(I:C) [31]. Thus, to assess the effect of viral infection on HAVCR2 expression in our studies, poly(I:C) was used to generate an *in vitro* model of viral infection in human fetal membranes and myometrium. Our studies show that *HAVCR2* mRNA and protein expression is significantly decreased in human fetal membranes and myometrium treated with poly(I:C). This altered expression of HAVCR2 in the presence of the viral mimetic poly(I:C) suggests HAVCR2 may have a role in preterm birth associated with viral infection.

There is now increasing evidence that viral infection induces inflammation in human gestational tissues. For example, infection with influenza virus upregulates secretion of IL6 and TNF in cultured primary human amnion and chorion cells [32]. Viral infection of myometrium and uterine cells are also associated with increased expression of pro-inflammatory mediators [19]. *In vitro* studies using human uterine cervical fibroblasts cells demonstrates influenza virus infection induces expression of IL1B, IL6, TNF and interferons (IFNA/IFNB) [33]. Of relevance to this study, we and others have also shown that the viral dsRNA analogue poly(I:C) induces the secretion of pro-inflammatory cytokines and pro-labour mediators in human fetal membranes [34] and myometrium [19]. Furthermore, administration of poly(I:C) induces preterm delivery via a TLR3-dependent manner within 24 h in pregnant mice [18]. It was thus of interest to determine if HAVCR2 regulates the expression of pro-inflammatory and pro-labour mediators induced by poly(I:C).

Cytokines and chemokines play a central role in human pregnancy and labour. The influx of leukocytes into the cervix and myometrium increases the production of pro-inflammatory cytokines and a congruent decrease in the production of anti-inflammatory cytokines in these tissues as well as fetal membranes [35]. These pro-inflammatory mediators promote the production of prostaglandins and matrix metalloproteases (MMPs) which activates cervical ripening, uterine contractions and fetal membrane rupture, leading to labour and delivery. In this study, loss-of-function experiments

using siRNA shows HAVCR2 regulates the inflammatory response to viral infection in human fetal membranes and myometrium. Specifically, poly(I:C)-stimulated expression of pro-inflammatory cytokines *TNF*, *IL1A*, *IL1B*, *IL6* and the chemokines *CXCL8* and *CCL2* was further elevated in siHAVCR2 transfected primary amnion and myometrial cells. HAVCR2 is a potent immunoinhibitory molecule with roles in immune tolerance and tumour or viral immune evasion [9]. In keeping with our studies, HAVCR2 has been shown to interact with TLRs to induce upregulated secretion of pro-inflammatory cytokines by innate immune cells [36]. Of relevance, HAVCR2 inhibits TLR4-mediated pro-inflammatory cytokine production in decidual stromal cells [25]. HAVCR2 blockade results in a significant increase in TLR-mediated production of the pro-inflammatory cytokine IL12, as well as a decrease in the immunoinhibitor molecule PDCD1 [14]. HAVCR2 activity suppresses Th1-mediated experimental autoimmune encephalomyelitis [9] and chronic hepatitis C infection is associated with HAVCR2 overexpression on $CD4^+$ and $CD8^+$ T cells [37]. Animal models have shown that post influenza infection, HAVCR2 is associated with a decrease in inflammatory cytokine expression [13], and that inhibiting HAVCR2 signalling decreased population regulatory T cells and increased inflammatory heart disease [38]. In corroboration, we found in the present study that loss of HAVCR2 by siRNA knockdown resulted in a significant increase in pro-inflammatory cytokines and chemokines as well as a significant decrease in anti-inflammatory cytokine production in response to poly(I:C). Of note, poly(I:C) stimulation of siCONT cells did not result in a significant decrease in anti-inflammatory cytokines *IL4* and *IL10* mRNA expression, which may suggest HAVCR2 could regulate anti-inflammatory cytokines irrespective of poly(I:C). However, in primary amnion cells, there was no difference in *IL4* and *IL10* mRNA expression between siCONT and siHAVCR2 transfected cells under basal conditions. There was an increasing trend in *IL4* and *IL10* mRNA expression in siHAVCR2 transfected myometrial cells under basal conditions, however this was not statistically significant. Nevertheless, taken together, these studies highlight the important role of HAVCR2 in mediating the inflammatory response in response to viral infection.

Prostaglandins are well described as potent stimulators of myometrium activation and contractility resulting in labour initiation. *PTGS2* is the main enzyme responsible for prostaglandin synthesis and is inducible by inflammation [39]. Prostaglandins, such as $PGF_{2\alpha}$, binds to its receptor, *PTGFR*, facilitating uterine contractions through increased calcium mobilisation; *PTGFR* mRNA expression is increased in human myometrium with term labour [40]. We have previously shown that poly(I:C) is a potent activator of the *PTGS2*-prostglandin pathway in human myometrium and fetal membranes [19, 23]. In this study, *HAVCR2* silencing significantly augmented poly(I:C)-stimulated *PTGS2* and *PTGFR* mRNA expression and release of $PGF_{2\alpha}$. This is the first study to show the involvement of

HAVCR2 in suppressing *PTGS2*-prostaglandin pathway activated by TLR3 in primary amnion cells, thus supporting the role of HAVCR2 as negative regulator of virus-induced labour onset.

Cell adhesion molecules play a critical role in the recruitment and chemotaxis of invading leukocytes to the myometrium, cervix and fetal membranes, resulting in increased inflammation and activation of terminal effector pathways that subsequently lead to human labour and delivery [41]. Although usually expressed on the cell surface, soluble forms of circulating ICAM1 and VCAM1 are detectable as a result of proteolytic cleavage processes. A recent study reported a positive association between elevated levels of sICAM1 and sVCAM1 and women who delivered preterm [42]. In the present study, expression and secretion of *VCAM1* and *ICAM1* in human myometrial and amnion cells were significantly increased with poly(I:C) treatment. This increase was significantly augmented with the loss of HAVCR2. To our knowledge, this is the first study to demonstrate HAVCR2 negatively regulates TLR3-mediated expression of adhesion molecules.

In accordance to the studies mentioned above, our studies indicate that HAVCR2 is required to suppress inflammation in the presence of viral infection. However, other reports have described an opposite role for HAVCR2, where the blockade of HAVCR2 is associated with enhanced T cell ability to clear chronic viral infection [43], tumours [44] and resolve autoimmune hepatitis [45]. Increased expression of HAVCR2 is also reported in peripheral blood mononuclear cells isolated from patients with hepatitis C virus (HCV) and HIV [46]. Thus, it appears that HAVCR2 can have opposing roles in innate and adaptive immunity. In human gestational tissues however, our data demonstrates that HAVCR2 plays an anti-inflammatory, protective role in TLR3-stimulated inflammation.

A limitation to this study was that we did not conduct HAVCR2 overexpression experiments to further elucidate the role of HAVCR2 in regulating inflammation in response to viral infection. These studies would determine if HAVCR2 overexpression could block the pro-inflammatory effects of poly(I:C) and complement our HAVCR2 siRNA data. Notwithstanding this limitation, this study does show loss of HAVCR2 function is associated with upregulated production of pro-inflammatory cytokines and pro-labour mediators in human gestational tissue in the presence of the viral dsRNA analogue and TLR3 ligand poly(I:C). It should also be noted that there was no effect of siHAVCR2 on pro-labour mediators in the presence of the pro-inflammatory cytokine IL1B or the TLR2 agonist and bacterial product fsl-1 (Supplementary Figure 3) suggesting that HAVCR2 is specifically required for TLR3 signalling. Another limitation of this study is that we did not assess the effect of other viral-sensing TLRs such as TLR7 and TLR8. Other studies have shown

activation of TLR7/8 can also significantly reduce HAVCR2 expression [47]. Unfortunately, we found no inflammatory response in tissues (fetal membranes, myometrium and placenta) nor in primary cells (myometrial and amnion cells) treated with ss40 (a ssRNA analogue) and thus have been unable to investigate the role of TLR7/8 on HAVCR2 function.

Studies in non-gestational tissues show poly(I:C) regulates HAVCR2 expression via activation of interferon response factor 3 (IRF3) [31]. We have previously reported IRF to play a key role in the terminal effector pathways of human preterm labour in primary myometrial cells. Thus, further studies on the effect of IRF3 on HAVCR2 function in myometrium is warranted to understand the mechanisms of preterm birth associated with viral infection.

To our knowledge, there are currently no commercially available HAVCR2 activators used for management of other inflammatory diseases. LGALS9 has been reported to be a ligand for HAVCR2. Interesting, LGALS9 is down-regulated in the chorion at the site of rupture [48]. Other studies have reported trophoblast cells express and secrete LGALS9, and via the LGALS9 / HAVCR2 signalling pathway, can induce natural killer (NK) cells to differentiate into decidual-like NK (dNK) cells [49]. These $HAVCR2^{+}$ dNK cells produce high levels of IL4 and low TNF. Therefore, it is feasible that LGALS9 could be used in the management of preterm birth by increasing HAVCR2 activation to reduce inflammation associated with labour.

By understanding and elucidating the mechanisms that drive human parturition, can we begin to develop new strategies that can delay and prevent preterm labour. In effect, it would improve preterm birth rates and lessen the unacceptably high mortality and morbidity rates associated with it. Viral infections can contribute to the intra-amniotic inflammation associated with preterm birth and leads to fetal injury and subsequent morbidities [50]. This study describes a novel role for HAVCR2 in the regulation of the inflammatory response in human fetal membranes and myometrium when stimulated with poly(I:C), the viral dsRNA analogue. Our findings show that HAVCR2 is normally expressed in human fetal membranes and myometrium, but in the presence of viral infection, HAVCR2 is suppressed. This loss of HAVCR2 results in the decreased production of anti-inflammatory cytokines (*IL4* and *IL10*) as well as the increased production of pro-inflammatory cytokines (*IL1A*, *IL1B*, *IL6* and *TNF*), chemokines (*CXCL8*, *CCL2*), activation of the *PTGS2*-prostglandin pathway and the upregulation of adhesion molecules (*ICAM1* and *VCAM1*). Thus, given that inflammation is an important aetiologic factor of preterm birth, the development of therapeutics that could increase HAVCR2 expression may be useful in the prevention spontaneous preterm birth associated with viral infections.

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AUTHORS' ROLES

ML conceived and designed the study, analysed the data and wrote the manuscript. RL and SL performed experiments, analysed the data and wrote the manuscript. GB performed experiments. All authors read and approved the final manuscript.

DISCLOSURE SUMMARY

The authors have nothing to declare.

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FIGURE LEGENDS

Figure 1. HAVCR2 expression in human fetal membranes and myometrium

Representative image demonstrating localisation of HAVCR2 in human **(A)** fetal membranes and **(B)** myometrium obtained from preterm (top) and term (bottom) women at the time of Caesarean section in the absence of labour. ae: amniotic epithelium; cl: connective tissue layer; ct: cytotrophoblast layer; dec: decidua. Arrows indicate HAVCR2 staining. Negative controls are also shown. Main picture magnification ×250; insert magnification ×100.

Figure 2. Effect of poly(I:C) on HAVCR2 protein expression in fetal membranes and myometrium

(A,B) Human fetal membranes and **(C,D)** myometrium were treated with 50 µg/ml poly(I:C) for 20 h. **(A,C)** *HAVCR2* mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to the basal group (n=5 patients). **(B,D)** HAVCR2 protein expression was analysed by Western blotting, normalised to β-actin protein expression and the fold change was calculated relative to the basal group (n=5 patients). All data is displayed as mean ± SEM. **P*<0.05 vs. basal (paired sample comparison). Representative Western blot from 1 patient is also shown.

Figure 3. Effect of siHAVCR2 on the expression and secretion of pro-inflammatory cytokines and chemokines in primary amnion cells

Human primary amnion cells were transfected with 200 nM siHAVCR2 or siCONT for 48 h and then treated with 5 µg/ml poly(I:C) for an additional 24 h (n=5 patients). **(A-F)** *TNF*, *IL1A*, *IL1B*, *IL6*, *CXCL8* and *CCL2* mRNA expression was analysed by qRT-PCR. **(G,H)** The incubation medium was assayed for concentration of IL6 and CXCL8 by ELISA. For all data, the fold change

was calculated relative to poly(I:C)-stimulated siCONT transfected cells. All data is displayed as mean ± SEM. *P<0.05 vs. poly(I:C)-stimulated siCONT transfected cells (one-way ANOVA).

Figure 4. Effect of siHAVCR2 on the expression and secretion of pro-inflammatory cytokines and chemokines in primary myometrial cells

Human primary myometrial cells were transfected with 200 nM siHAVCR2 or siCONT for 48 h and then treated with 5 µg/ml poly(I:C) for an additional 24 h (n=5 patients). **(A-F)** *TNF*, *IL1A*, *IL1B*, *IL6*, *CXCL8* and *CCL2* mRNA expression was analysed by qRT-PCR. **(G,H)** The incubation medium was assayed for concentration of IL6 and CXCL8 by ELISA. For all data, the fold change was calculated relative to poly(I:C)-stimulated siCONT transfected cells. All data is displayed as mean ± SEM. *P<0.05 vs. poly(I:C)-stimulated siCONT transfected cells (one-way ANOVA).

Figure 5. Effect of siHAVCR2 on the expression of anti-inflammatory cytokines

(A,B) Human primary amnion cells and **(C,D)** human primary myometrial cells were transfected with 200 nM siHAVCR2 or siCONT for 48 h and then treated with 5 µg/ml poly(I:C) for an additional 24 h (n=5 patients). **(A,D)** *IL4* and **(B,D)** *IL10* mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to poly(I:C)-stimulated siCONT transfected cells. All data is displayed as mean ± SEM. *P<0.05 vs. poly(I:C)-stimulated siCONT transfected cells (one-way ANOVA).

Figure 6. Effect of siHAVCR2 on the prostaglandin pathway

(A-C) Human primary amnion cells and **(D-F)** human primary myometrial cells were transfected with 200 nM siHAVCR2 or siCONT for 48 h and then treated with 5 µg/ml poly(I:C) for an additional 24 h (n=5 patients). **(A,B,D,E)** *PTGS2* and *PTGFR* mRNA expression was analysed by qRT-PCR. **(C,F)** The incubation medium was assayed for concentration of $PGF_{2\alpha}$ by ELISA. For all data, the fold change was calculated relative to poly(I:C)-stimulated siCONT transfected cells. All data is displayed as mean ± SEM. *P<0.05 vs. poly(I:C)-stimulated siCONT transfected cells (one-way ANOVA).

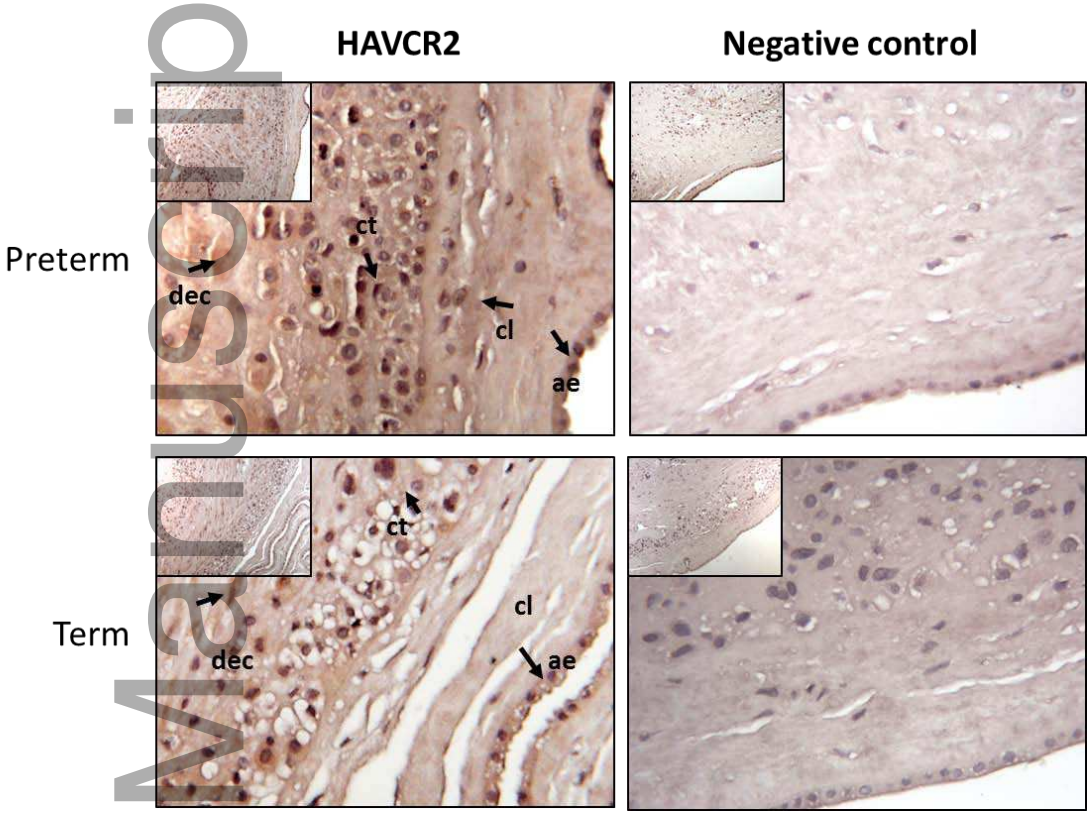
Figure 7. Effect of siHAVCR2 on the expression and secretion of adhesion molecules

(A-D) Human primary amnion cells and **(E-H)** human primary myometrial cells were transfected with 200 nM siHAVCR2 or siCONT for 48 h and then treated with 5 µg/ml poly(I:C) for an additional 24 h (n=5 patients). **(A,E)** *ICAM1* and **(C,G)** *VCAM1* mRNA expression was analysed by qRT-PCR. The incubation medium was assayed for concentration of **(B,F)** sICAM1 and **(D,H)** sVCAM1 by ELISA. For all data, the fold change was calculated relative to poly(I:C)-stimulated

siCONT transfected cells. All data is displayed as mean ± SEM. *P<0.05 vs. poly(I:C)-stimulated siCONT transfected cells (one-way ANOVA).

Figure 1

A)



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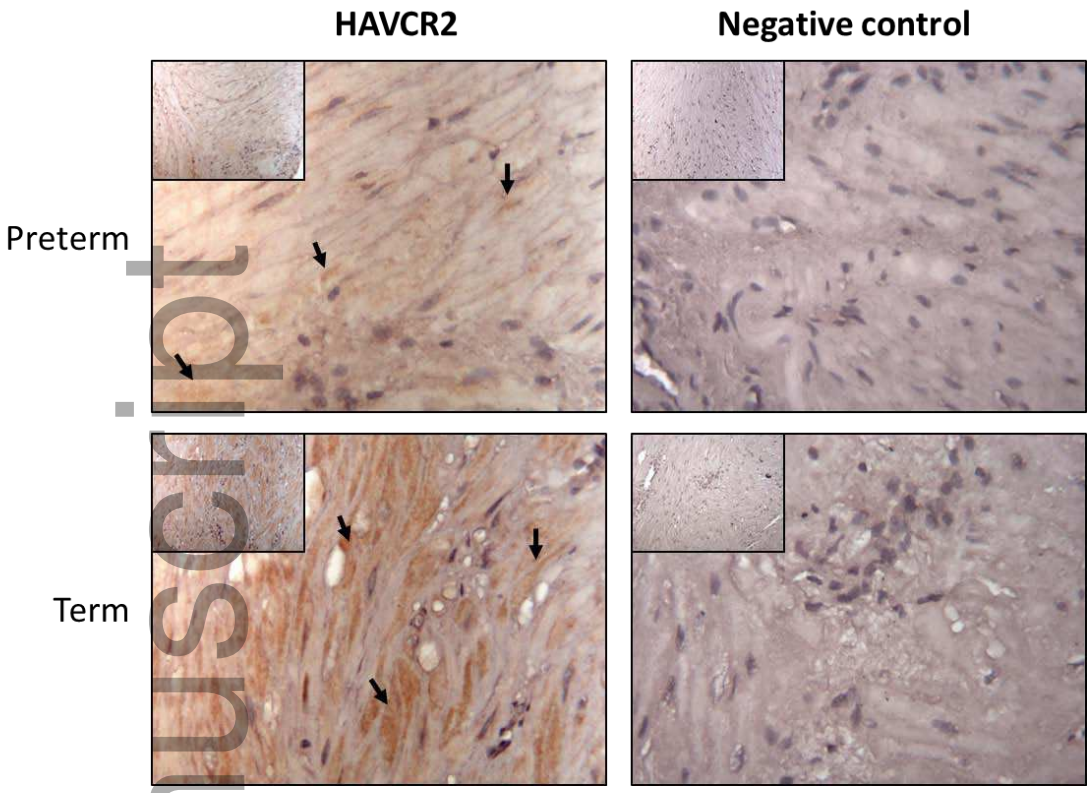

Figure 2

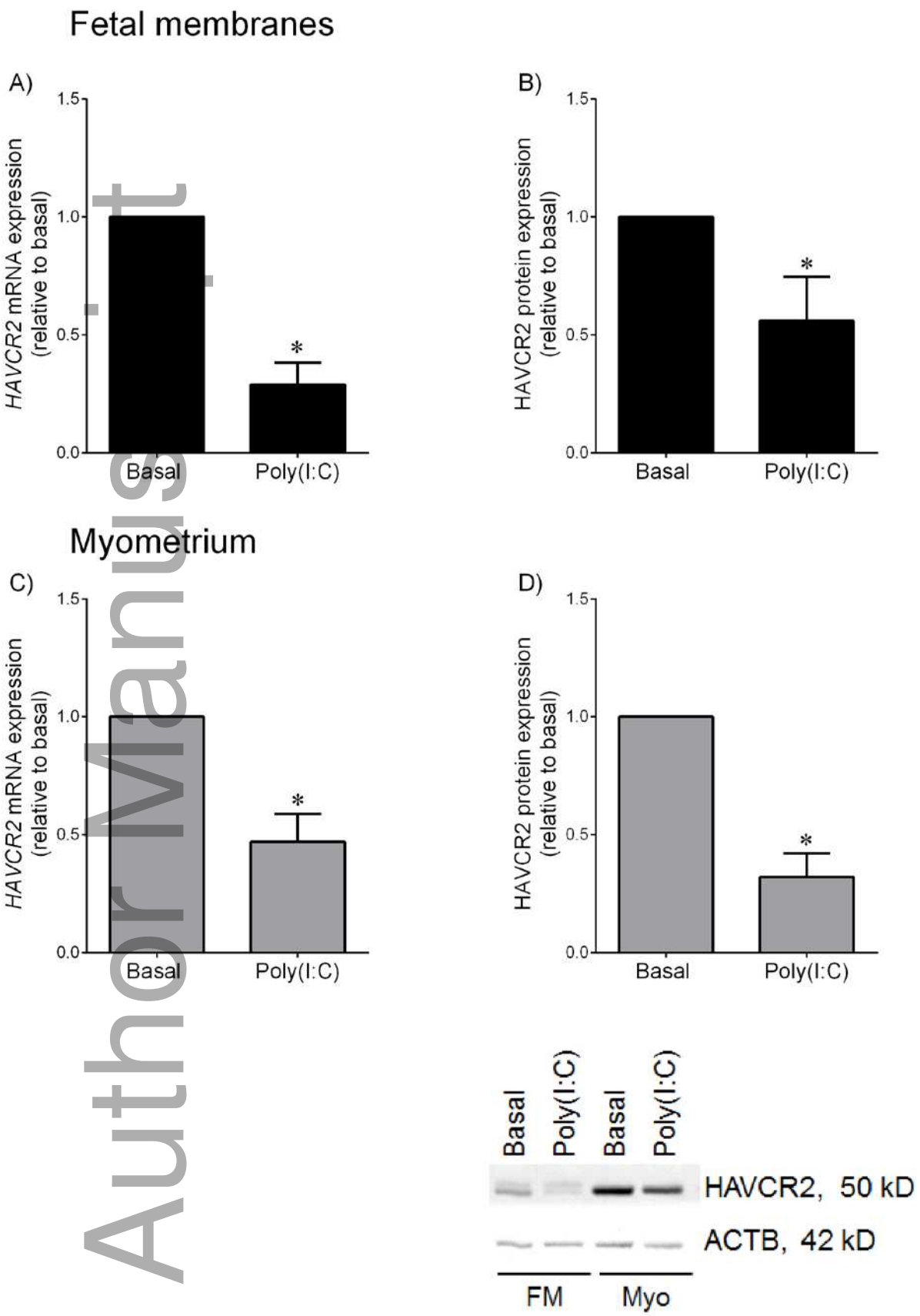

Figure 3

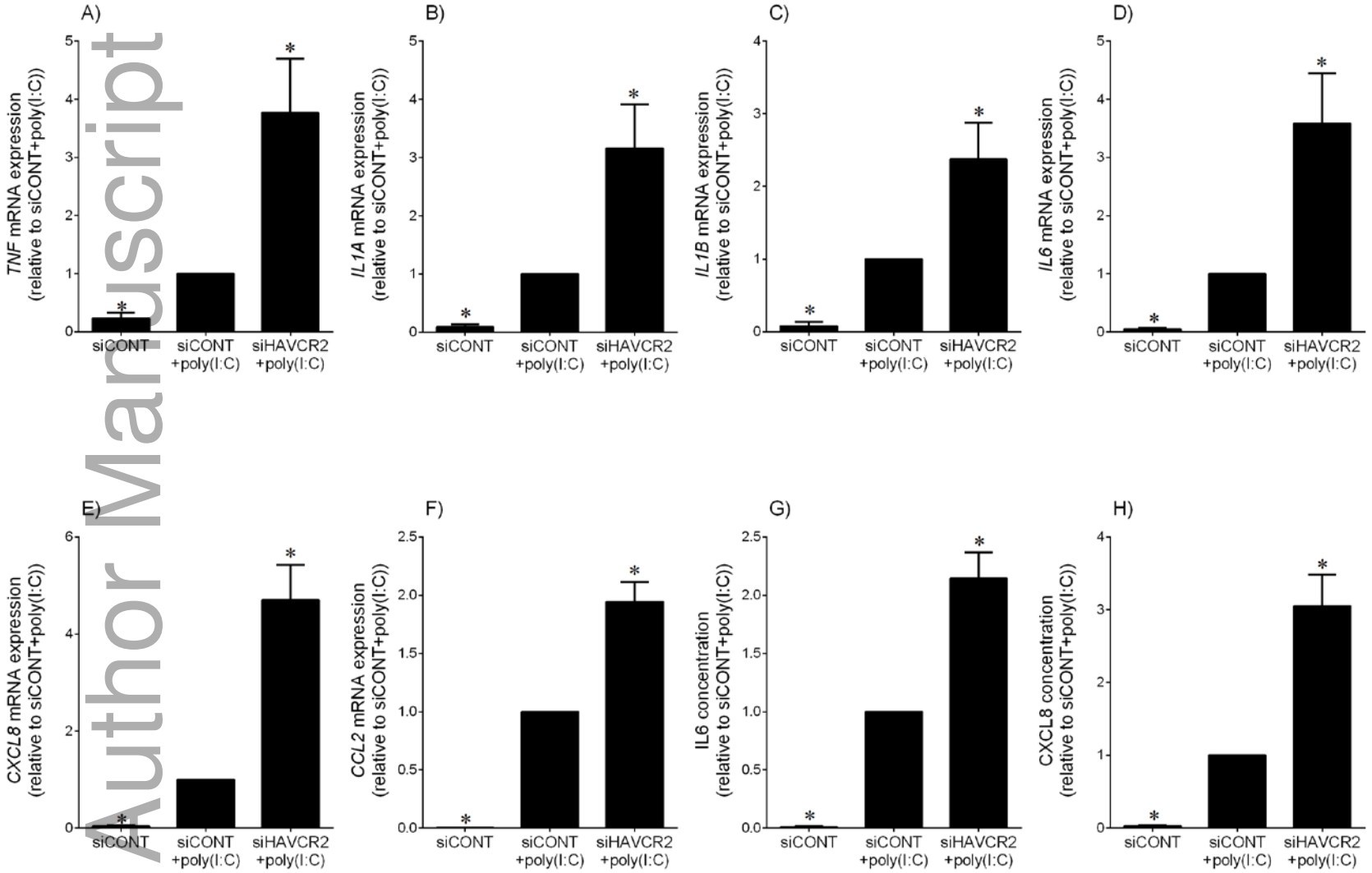

Figure 4

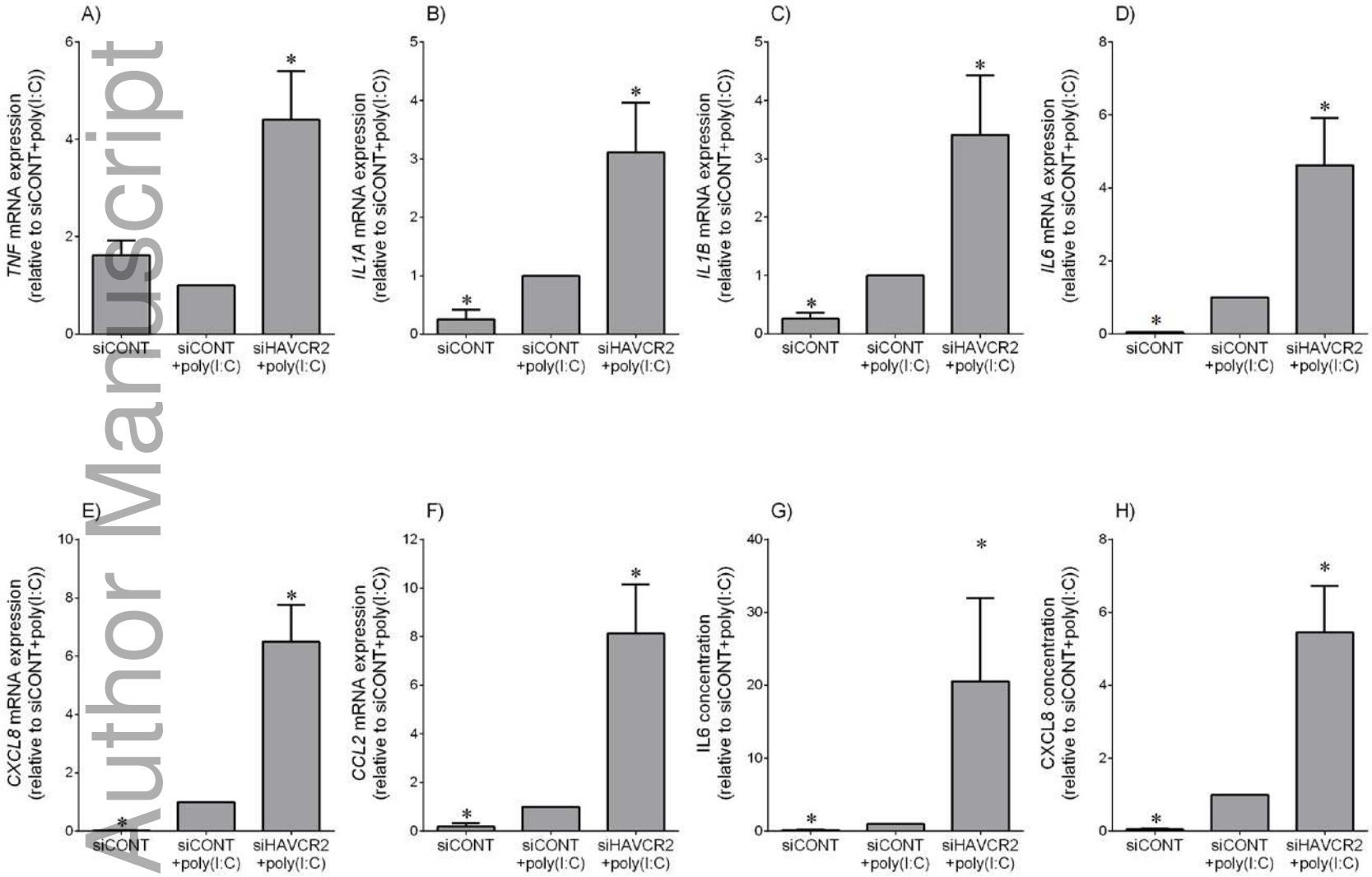

Figure 5

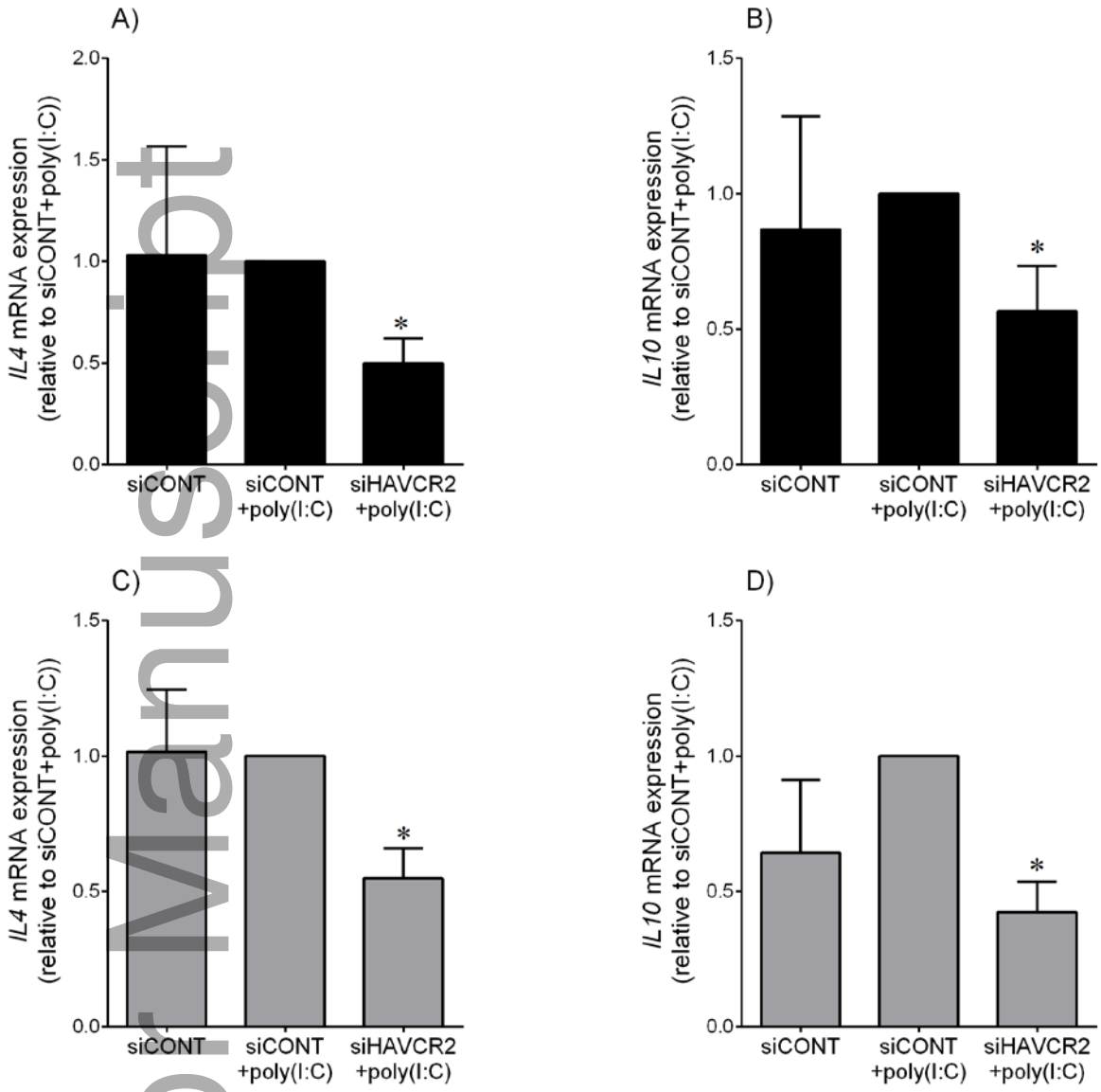

Figure 6

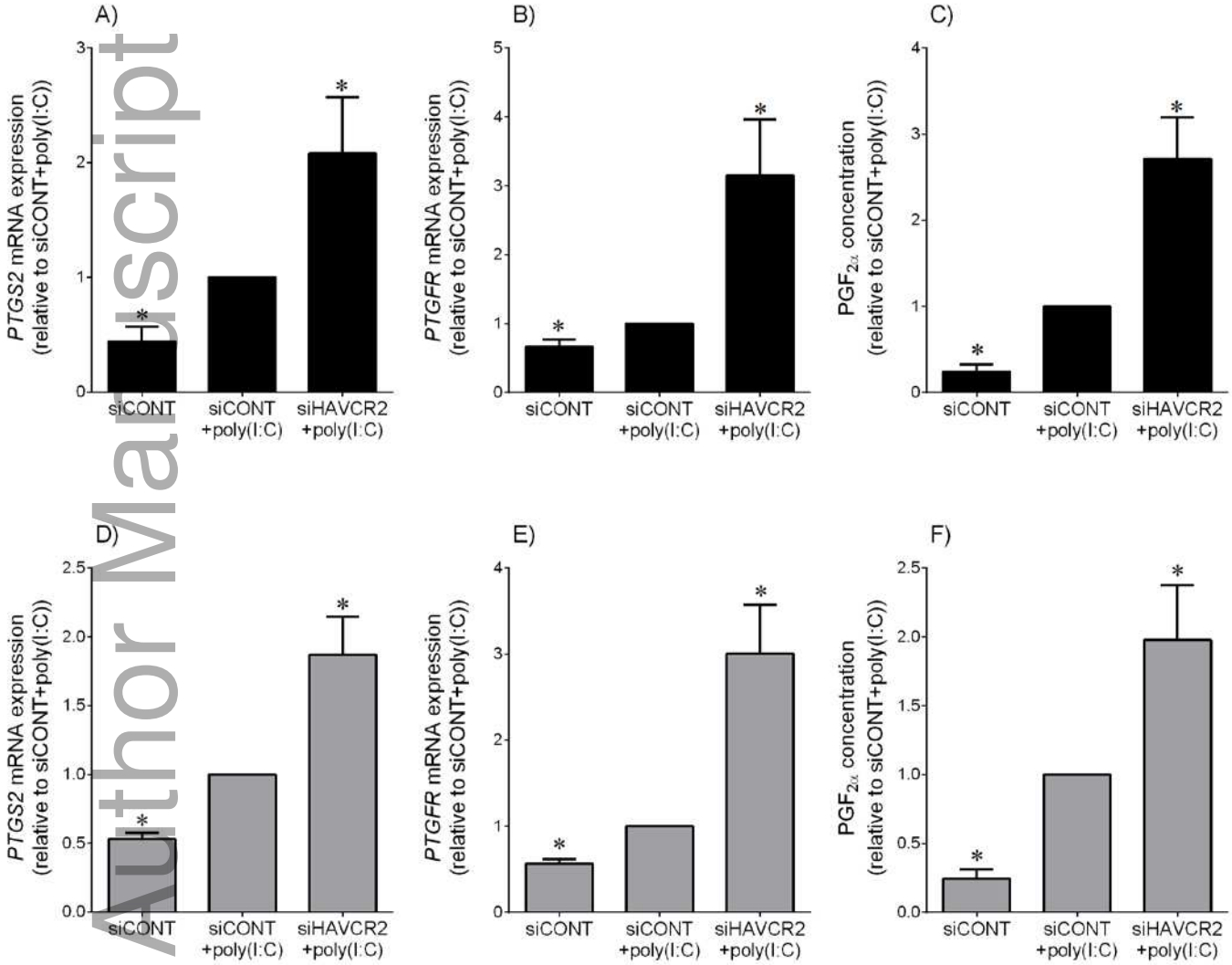

Figure 7