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Role of endoplasmic reticulum stress in pro-inflammatory cytokine–mediated inhibition of trophoblast invasion in placenta-related complications of pregnancy

Cheuk-Lun Lee[1,2,3], Jan H.W. Veerbeek[4], Tirtha K. Rana[1], Bas B. van Rijn[4]

Graham J. Burton[1] and Hong Wa Yung[1]*

Footnote: C-L.L., J.H.W.V., and T.K.R. contributed equally.

[1]Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, CB2 3EG. [2]Department of Obstetrics and Gynaecology, LKS Faculty of Medicine, The University of Hong Kong. [3]Shenzhen Key Laboratory of Fertility Regulation, Department of Obstetrics and Gynecology, The University of Hong Kong-Shenzhen Hospital. [4]Department of Obstetrics and Gynaecology, Division of Woman and Baby, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands.

* To whom correspondence should be addressed: Dr Hong Wa Yung, Tel: +44 1223 333862. Fax: +44 1223 333840, e-mail: hwy20@hermes.cam.ac.uk.

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Abstract

Shallow extravillous trophoblast (EVT) invasion is central to the pathophysiology of many pregnancy complications. Invasion is mediated partially by matrix metalloproteinases (MMPs). MMP-2 is highly expressed in early pregnancy. MMP activity can be regulated by pro-inflammatory cytokines, which also induce endoplasmic reticulum (ER) stress in other cells. We investigated whether pro-inflammatory cytokines regulate MMP-2 activity through ER stress response pathways in trophoblast, before exploring potential regulatory mechanisms. There was increased immunoreactivity of HSPA5, also known as GRP78, in cells of the placental bed, including EVTs, in cases of early-onset preeclampsia compared to normotensive controls. Treating EVT-like JEG3 and HTR8/SVneo cells with ER stress inducers, tunicamycin and thapsigargin, suppressed *MMP2* mRNA and protein expression, secretion, activity, and reduced their invasiveness. A cocktail of pro-inflammatory cytokines, interleukin-1β, tumor necrosis factor-α, and interferon-γ, suppressed MMP-2 activity in JEG-3 cells, and was accompanied by activation of the PERK-EIF2A arm of the ER stress pathway. Knockdown of *ATF4*, a downstream transcriptional factor of the PERK-EIF2A pathway, by small interference RNA, restored *MMP2* expression but not cellular proteins. However, suppression of EIF2A phosphorylation with a PERK inhibitor, GSK2606414, under ER stress, restored MMP-2 protein. ER stress regulates MMP-2 expression at both the transcriptional and translational levels. This study provides the

first mechanistic linkage by which pro-inflammatory cytokines may modulate trophoblast invasion through ER stress pathways.

Introduction

The invasion of extravillous trophoblast (EVT) into the decidualized endometrium is crucial in the determination of pregnancy outcome. Inadequate trophoblast invasion not only leads to implantation failure [1] and spontaneous pregnancy loss, but also results in the insufficient remodeling of spiral arteries that sits at the epicenter of the 'Great Obstetrical Syndromes', including idiopathic fetal growth restriction (FGR) [2], early-onset pre-eclampsia [3, 4] and preterm birth [5, 6]. The EVTs invade soon after implantation and complete the process around mid-gestation, penetrating as far as the inner one-third of the myometrium. Although many factors and biomolecules such as TGFβ, kisspeptin, hypoxia, and the interaction with immune cells, have been proposed to regulate the invasiveness of the EVTs, their downstream effectors principally converge on a family of matrix metalloproteinase enzymes (MMPs), that break down both matrix and non-matrix proteins [7-9]. MMP-2 and MMP-9 are likely two key players. MMP-2 mediates trophoblast invasion during the early implantation stage up to seven to eight weeks of gestation, whereas MMP-9 facilitates subsequent invasion [10-13]. Although the regulation of MMP activity has been widely studied, the mechanisms remain largely unknown.

MMPs are controlled at multiple levels. Transcriptional regulation occurs upon stimulation by a variety of pro-inflammatory cytokines, growth factors, and hormones, as well as by interactions between cells or between cells and their surrounding matrix [14].

MMPs are synthesized as precursor zymogens, and are post-translationally modified and folded within the endoplasmic reticulum (ER) before either extracellular export or transport to the plasma membrane. Their activation is dependent on sequential proteolysis of the propeptide that blocks the active site, and is regulated by a number of factors including plasmin, MMP intermediates and other active MMP family members [15]. Furthermore, MMP activity can be modulated by exogenous inhibitors, such as α2-macroglobulin and a group of tissue inhibitors of metalloproteinases (TIMPs) [16]. The requirement for proteolytic cleavage implies that the conformation of the MMPs is critical for their activation. Hence, post-translational modifications, such as glycosylation and disulphide bond formation, may serve as novel regulatory pathways under stress conditions that are known to trigger ER stress or the ER unfolded protein response (UPR^{ER}). All three UPR^{ER} signaling pathways PERK, ATF6, and IRE1, can regulate gene expression directly through their downstream transcriptional factors ATF4/CHOP, cleaved ATF6, and spliced XBP-1, respectively [17]. For example, we have demonstrated that expression of placental growth factor (PlGF) is mediated through ATF4 and ATF6β signaling in placenta of early-onset pre-eclampsia [18].

Pro-Inflammatory cytokines have been demonstrated to suppress trophoblast migration [8], invasion [19], and integration [20], resulting in deficient spiral artery remodeling [21-23]. The major source of pro-inflammatory cytokines in the decidua is the immune cells,

of which ~70% are decidual natural killer (dNK) cells and ~20% are macrophages [24]. dNK cells have a unique phenotype and properties compared to their peripheral blood counterparts, and secrete cytokines and other soluble factors to modulate implantation, placental function and ultimately fetal development. Aberrant behavior of these cells has been suggested to contribute to the pathogenesis of pre-eclampsia [25-30]. However, the mechanisms by which these cytokines inhibit trophoblast invasion remain unknown [31]. Coincidently, pro-inflammatory cytokines also induce ER stress in other mammalian cell types [32-34]. Therefore, we investigated the potential role of ER stress in the regulation of trophoblast MMP-2 activity, thereby modulating EVT invasion during early pregnancy.

The potential existence of ER stress was first examined in EVTs in placental bed biopsies obtained from pregnancies complicated by early-onset pre-eclampsia. It was then tested whether ER stress can modulate MMP-2 activity before elucidating the role of pro-inflammatory cytokines in the induction of ER stress and suppression of MMP-2 activity in extravillous-like trophoblastic cells. Finally, the molecular mechanisms by which the UPR^{ER} pathways may regulate MMP-2 activity were explored..

Materials and Methods

Immunohistochemistry

The ethical approval, the criteria diagnosis of early-onset pre-eclampsia and the

procedures for the placental bed biopsies were described previously [35]. Immunohistochemistry of cytokeratin 7 (CK7) and HSPA5/GRP78 were performed on paraffin-embedded placental villous and placental bed sections (5 μm) using antibodies to CK7 (Dako, Agilent Technologies LDA UK Limited, Stockport, UK) and HSPA5 (Abcam, Cambridge, UK).

Cell cultures

The human choriocarcinoma cell line, JEG-3, and the HTR8/SVneo trophoblast cell line were cultured in RPMI 1640 medium (Invitrogen Ltd, Paisley, UK) containing 10% heat-inactivated FBS (Invitrogen), 100U/mL penicillin, and 100μg/mL streptomycin at 37 ℃ in 5% CO_2 atmosphere. Both cell lines express markers of EVT [36]. Upon reaching confluence, cells were dissociated from the culture flask using 0.05% Trypsin-EDTA (Invitrogen) for sub-culture or further experiments.

For experiments, JEG-3 and HTR8/SVneo cells were seeded at $1.25x10^5$ cells/mL and $2x10^5$ cells/mL, respectively, in 2 mL of culture medium in a 6-well plate for 48 h before treatment. Cells were rinsed with serum-free medium twice before application of cytokines or drugs in 1 mL serum-free medium for 24 h. All cytokines were used at 50 ng/mL. For JEG-3 cells, tunicamycin was used at 0.31, 0.62, 1.25, and 2.50 μg/mL for dose-response study and thapsigargin was at 100 nM. For HTR8/SVneo cells, tunicamycin and thapsigargin were used at 78 ng/mL and 125 nM, respectively.

GSK2606414 was used at 100 nM and purchased from Generon (Slough, UK). All other drugs, chemicals, and cytokines were purchased from Sigma (Sigma-Aldrich Company Ltd, Dorset, UK).

Immunofluorescence

After treatment, cells were fixed with 4% paraformaldehyde in PBS for 20 min. After washing, the cells were incubated with blocking buffer containing 1% bovine serum albumin (BSA) (Sigma) and 0.1% saponin (Sigma) in PBS for 30 min before incubating with primary antibody against ATF4 (New England Biolabs, Hitchin, UK) for overnight at room temperature. After washing, cells were incubated with secondary antibody conjugated with fluorescein Alexa 488 (Vector Laboratories, Peterborough, UK) for 1 h followed by staining in 5 μg/mL Hoechst 33342 (Sigma) nuclear dye staining for 10 min. Images were taken by EVOS FL Color Imaging system (Life Technologies) at 200X magnification.

***ATF4* gene knockdown**

Expression of *ATF4* was suppressed by RNA interference using Lipofactamine RNAiMax (Invitrogen) as described by the manufacturer. In brief, JEG-3 cells (2.5×10^5) were plated in a 6-well plate. The day before reaching ~30% to 40% confluence they were transfected with 30 pmol of siRNA molecules targeted against human *ATF4*

(Sigma, SAS1_Hs02_00332313) or control siRNA molecules (Sigma, SIC001) using 9 µL of Lipofactamine RNAiMax (Invitrogen) in 300 µL of OptiMEM medium (Invitrogen) for 48 h. The wells were rinsed with serum-free medium and incubated with 1 mL of serum-free medium containing 100 nM of thapsigargin (Sigma) for 24 h.

Protein sample preparation and western blot

Protein isolation and western blotting analysis were performed as previously described [37]. Primary antibodies for ATF4, EIF2A, Phospho-EIF2A(Ser51), and MMP-2 were from Cell Signaling Technology (New England Biolabs, Hitchin, UK); HSPA5/GRP78 from Transduction Lab (BD Biosciences, Wokingham, UK); and TIMP1 from Abcam. The results are shown as

Relative Ratio (%) = (Density of treatment group/Density of control) x 100%.

Zymogen assay for MMP-2 activity

MMP-2 activity was determined by gelatin gel zymography. In brief, the conditioned media were concentrated and equal volumes were used for native gel electrophoresis. After resolving, the gel was incubated in 2.5% Triton X-100 for 1 h before incubation in buffer containing 10 mM $CaCl_2$, 200 mM NaCl, and 50 mM Tris HCl overnight at 37 ℃. The gel was stained with PAGE b lue (ThermoFisher scientific, UK)

overnight before destaining with water.

Trophoblast Invasion Assay

Invasive potential was determined by transwell invasion assay using an 8 µm insert (BD Biosciences, Wokingham, UK). In brief, HTR8/SVneo cells ($1x10^5$/well) in serum-free culture medium were placed in the upper chamber, while the lower chamber contained culture medium with 10% FBS. After 24 h, the medium and the cells in the upper chamber were discarded. Cells that had invaded through to the undersurface of the membrane were fixed and permeabilized with ice-cold methanol, stained with 0.6 µg/mL SYTOX-green (S33025, Invitrogen) and visualized with a Litz DM1L microscope (Leica Microsystems, Wetzlar, Germany). The number of cells was quantified using ImageJ version1.51h. The results are shown as

rate of invasion (%) = (Invasiveness of treatment group/Invasiveness of control) x 100%.

Reverse transcription and quantitative reverse transcription-PCR

Total RNA was isolated using Qiagen RNeasy Mini Kits (Qiagen Ltd, Manchester, UK) following the manufacturer's instructions. cDNA synthesis was performed as previously described [37]. Both TaqMan and SYBR Green were used to quantify gene

expression. For TaqMan, the probes were *MMP2* (Hs01548727_m1), *GAPDH* (Hs99999905_m1), and *18S* (Hs99999901_s1) using TaqMan Gene Expression Assays (Applied Biosystems). For SYBR Green, the primers were *ATF4* (Sense: 5'-GACGGAGCGCTTTCCTCTT-3'; Antisense: 5'-TCCACAAAATGGACGCTCAC-3'); *18S* (Sense: 5'-GTAACCCGTTGAACCCCATT-3'; Antisense: 5'-CCATCCAATCGGTAGTAGCG-3'); *TBP* (Sense: 5'-GTGGGGAGCTGTGATGTGA-3'; Antisense: 5'-AATAAGGAGAACAATTCTGGTTTG-3') and were analyzed by SYBR Green JumpStart kits (Sigma). Gene expression levels were determined using the threshold cycle method ($2^{-\Delta\Delta CT}$ method) with reference to the endogenous controls of either *18S* and *GAPDH* or *18S* and *TBP*. The results are presented as relative expression.

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Differences between study groups were analyzed by the One-way ANOVA test or paired two-tailed non-parametric Friedman test when appropriate. Correlation analysis was conducted using Pearson's test. $P < 0.05$ was considered significant. Statistical analysis was performed using either the Statistical Package for the Social Sciences version 21.0 (IBM Corp) or Prism GraphPad version 6.0.

Results

Existence of ER stress in the extravillous trophoblast (EVT) in placental bed samples from pre-eclamptic pregnancies

The study by Lian and colleagues demonstrated an increase of ER stress in the decidual tissues of pre-eclamptic patients [38]. Therefore, immunohistochemistry was performed for HSPA5 (also known as glucose-regulated protein, GRP78) and cytokeratin 7 (CK7) on serial sections to confirm the existence of high level of ER stress in EVTs within placental bed samples collected from pre-eclamptic and normotensive pregnancies after caesarean delivery. There was no statistical significant difference in maternal age and maternal BMI between two groups (Table 1), but there were significant ($P < 0.001$) differences in systolic and diastolic blood pressure, gestational age, and birth weight. There were many CK7-positive EVTs in both pre-eclamptic and normotensive control pregnancies (Figure 1). Increased HSPA5 staining in the pre-eclamptic compared to the normotensive samples, confirmed a higher degree of ER stress in those cases (Figure 1). ER stress was not restricted solely to the EVTs, and decidual cells also showed strong immunoreactivity, as in the study of Lian et al[38]. It was also noteworthy that the staining was not even across the entire pre-eclamptic placental bed, indicating the existence of regional variations (data not shown).

Induction of ER stress suppresses MMP-2 expression, secretion, and activity in

trophoblast

Invasion of EVTs into the endometrium and inner one-third of the myometrium is a crucial stage in human placentation, and is mainly mediated by MMP-2 in the first trimester [12, 13, 39]. It was therefore investigated whether ER stress can directly alter MMP-2 activity using the ER stress inducer tunicamycin and extravillous-like trophoblast JEG-3 cells. Tunicamycin triggered a dose-dependent increase in severity of ER stress in JEG-3 cells as demonstrated by the biomarkers, P-EIF2A, ATF4, HSPA5, and GRP94 (Figure 2A), which were increased by 533% ± 88%, 965% ± 204%, 288% ± 41%, and 302% ± 141%, respectively, at 1.25 μg/mL (Figure 2A). Crucially, tunicamycin treatment also reduced the cellular levels, secretion, and activity of MMP-2 in a dose-dependent manner (Figure 2B). At 1.25 μg/mL these were suppressed by 21.0% ± 5.7%, 15.2% ± 4.1%, and 37.5% ± 10.3%, respectively (Figure 2B). To investigate whether the inhibition of cellular MMP-2 operates at the transcriptional or translational level, *MMP2* transcripts were assayed by quantitative reverse transcription-PCR. A dose-dependent reduction was observed upon tunicamycin treatment (Figure 2C).

Induction of ER stress inhibits trophoblast invasion

To eliminate drug- and cell-specific effects, another trophoblast cell line, the first trimester immortalized HTR8/SVneo cell, and an additional ER stress inducer, thapsigargin, were introduced. Both thapsigargin and tunicamycin stimulated higher

levels of P-EIF2A, HSPA5, and GRP94 (Figures 3A and B), and reduced secretion and activity of MMP-2 (Figures 3C and D). Additionally, the secreted form of TIMP1, an extracellular inhibitor of MMP-2, was also reduced, indicating that the loss of MMP-2 activity is unlikely mediated by the TIMP1 regulatory mechanism (Figure 3C). Finally, a transwell invasion assay was used to demonstrate the effect of ER stress on HTR8/SVneo cell invasion. Treatment with the ER stress inducers caused a >70% reduction in cells penetrating through the Matrigel as compared to controls, confirming reduced invasive capacity (Figures 3E and F).

Inhibition of trophoblast MMP-2 expression, secretion, and activity by pro-inflammatory cytokines is accompanied by induction of ER stress

Pro-inflammatory cytokines, which can result from infection or systemic inflammation, are known to inhibit trophoblast invasion. They also trigger ER stress in other human cells, as well as in villous-like trophoblast BeWo cells [33, 40, 41]. Therefore, three pro-inflammatory cytokines, IL-1β, TNF-α, and IFN-γ were administered to JEG3 cells. Western blotting analysis showed that cells treated with all three cytokines showed significantly reduced levels of cellular MMP-2 (33.7% ± 2.9%), secretion (41.8% ± 4.0%), and activity (43.5% ± 8.0%), compared with the control group (Figures 4A and B). By contrast, JEG-3 cells treated with individual cytokines showed only very subtle or no effects (Figure 4B).

Next, it was investigated whether the pro-inflammatory cytokines were able to induce ER stress in JEG-3 cells. Treatment with TNF-α or all cytokines together increased levels of phosphorylated EIF2A (P-EIF2A), a marker of ER stress, significantly by 148.2% ± 16.5% and 168.4% ± 21.0%, respectively, compared to controls (Figure 4C and D). The ER chaperone HSPA5 remained unchanged, indicating only low-grade ER stress (Figures 4C and D). Correlation analysis between P-EIF2A and MMP-2 (cellular, secreted, and active forms) showed very strong inverse relationships (R^2=0.58, 0.70, and 0.98, respectively) (Figure 4E). These results indicated potential regulation of MMP-2 by the PERK-EIF2A arm of the UPR pathway.

ATF4 and EIF2A negatively regulate *MMP2* transcription and translation in response to ER stress

ATF4 is a transcription factor downstream of the PERK-EIF2A arm of the UPR signaling pathway. Therefore, its potential role in regulating *MMP2* transcription was investigated. A negative correlation (R^2=0.88 and 0.91) between the levels of ATF4 and cellular or secretory MMP-2 was observed in JEG3 cells (Figure 5A). These results suggested potential regulation of *MMP2* transcription by ATF4, and therefore small RNA interference was used to knockdown *ATF4* transcripts before treatment with thapsigargin.

siATF4 treatment abolished the rise in ATF4 induced by thapsigargin (Figures 5C and D). As expected, knockdown of *ATF4* gene induced a 2.2-fold increase of *MMP2* mRNA level in *siAFT4*-treated cells in both the presence or absence of thapsigargin (Figure 5B). However, the increased *MMP2* transcripts failed to translate into protein as the cellular MMP-2 level was still reduced by 65% in *siATF4*-transfected cells. Elevation of *MMP2* transcripts also failed to restore cellular MMP-2 protein levels upon treatment with thapsigargin, which still showed a 49% reduction in the *siATF4*-transfected cells compared to *siCon*-transfected cells (Figure 5C and D). PERK-mediated phosphorylation of EIF2A causes attenuation of non-essential protein translation [42]. To investigate the potential translational regulation of MMP-2 by PERK-EIF2A, a PERK inhibitor, GSK2606414, was added in the presence and absence of the ER stress inducers tunicamycin or thapsigargin Application of GSK2606414 reduced ER stress-mediated phosphorylation of EIF2A and restored MMP-2 protein level (Figure 5E). However, the ATF4 level was maintained at a high level under the treatment. Nuclear localization is crucial for transcriptional regulation. Therefore, ATF4 cellular localization was examined by immunocytochemistry. Indeed, ATF4 nuclear localization was largely absent in the presence of GSK2606414 whereas there was clearly nuclear staining of ATF4 in untreated cells upon ER stress (Figure 5F). These results strongly suggest that the expression of MMP-2 is regulated both transcriptionally and

translationally by PERK-EIF2A-ATF4 pathway in response to ER stress.

Discussion

Endoplasmic reticulum stress has been demonstrated in both the placenta and decidua in cases of idiopathic FGR and early-onset pre-eclampsia [38, 43]. In the placenta, the stress is likely a consequence of hypoxia-reperfusion injury triggered by insufficient remodeling of the spiral arteries. However, the trigger for the decidual stress is unknown, and its impact on pregnancy outcome has not been explored. In this study, pro-inflammatory cytokines were identified as a potential source of decidual ER stress, and the inhibitory role ER stress exerts on trophoblast invasion through modulation of MMP-2 activity was demonstrated. Furthermore, our results elucidated that the PERK arm of UPR signaling in ER can directly regulate MMP-2 at both the translational and transcriptional levels by PERK-EIF2A and ATF4, respectively. Overall, these results provide new insights into the molecular pathology that may underpin cases of reduced trophoblast invasion, and hence spiral artery remodeling, in pregnancy complications.

Several pre-gestational pathological conditions result in aberrant increases of pro-inflammatory cytokines within the uterine cavity, and are associated with an increased risk of pre-eclampsia. For example, infection with *Chlamydia trachomatis* increases levels of IFN-γ in cervical secretions [44], the secretion of IL-1β and TNF-α in

dendritic cells [45], and the risk of pre-eclampsia [46]. Elevated levels of uterine pro-inflammatory cytokines likely induce ER stress in the invading EVTs, in a similar fashion to the way that injection of IL-1β and IL-6 induces ER stress in pancreatic Islet cells [47]. Compromise of MMP activity will inhibit invasion into the deeper regions of the endometrium and myometrium, and the clinical outcome will be dependent on the severity of the subsequent deficit in arterial remodeling. Milder cases will result in pre-eclampsia and/or FGR, whereas severe cases will end in miscarriage. The severity of ER stress determines trophoblast cell fate; at low levels it reduces cell proliferation whereas at high levels it induces apoptosis [43].

The literature regarding regulation of trophoblast invasion by pro-inflammatory cytokines is contentious. Although many studies have revealed their inhibitory role, there is also evidence that the same cytokines may promote trophoblast migration and invasion [22, 23, 48, 49]. In normal pregnancy, IL-1β (1 to 10 ng/mL) up-regulates the proteases MMPs and urokinase type plasminogen activator systems to promote trophoblast motility [48, 49], whereas decidual NK cell–derived IFN-γ is necessary for spiral artery remodeling and placental formation [22, 23]. On the other hand, inhibition of trophoblast invasion by IFN-γ associated with reduced secretion of MMP-2 has been reported [19]. Additionally, TNF-α inhibits trophoblast migration [8] and integration into maternal endothelial cellular networks, which also involves the inhibition of MMP-2 [20]. In

a rat model, TNF-α is causally linked to deficient trophoblast invasion and spiral artery remodeling leading to features of pre-eclampsia and fetal growth restriction [21]. These results reveal the complexity of the regulation of trophoblast invasion by pro-inflammatory cytokines. Although the mechanisms behind these opposite roles are unknown, the concentration of the cytokines, their spatial and temporal profiles, sources/origins (immune cells or endometrial cells), and interactions with other cytokines may explain the differences [50, 51]. Changes in pro-inflammatory cytokine profiles may also alter the interactions between dNK cells and trophoblast cells, thereby modulating the invasion process [52]. The local milieu is therefore likely to be critical, but mimicking the precise conditions within the decidua *in vivo* is difficult in reductionist experimental situations. For example, TNF-α and IFN-γ, but not IL-1β, inhibited MMP-2 activity when administered individually, whereas a mixture of all three cytokines produced a synergistic effect (Figure 4). Crucially, similar effects were also observed in their capacity to activate phosphorylation of EIF2A, the PERK arm of UPR pathway in ER.

Pro-inflammatory cytokines induce ER stress in many mammalian cell systems [32-34]. Our results demonstrate that these cytokines also trigger ER stress in trophoblast JEG-3 cells. However, the severity of stress is likely low-grade because only the PERK-EIF2A arm of the UPR^{ER} pathway was activated. Similar low-grade ER stress

was observed in the trophoblast cells of the human placenta following pregnancy at high altitude [53], and in the mouse placenta upon hypoxic challenge [54]. It has been suggested that the severity of ER stress induced by the pro-inflammatory cytokines can be cell-type specific and also species specific [33]. The mechanisms by which they activate UPR pathways in ER are unclear, but several studies suggest both direct activation and indirect activation mediated by nitric oxide (NO) or perturbation of calcium homeostasis. A mixture of IL-1β, TNF-α, and IFN-γ induces splicing of X-box binding protein 1 (XBP1), a downstream effector of the IRE1α arm of the UPR^{ER} pathway, and phosphorylation of Eif2A in mouse islet and MIN6 cells independent of NO production [55]. Conversely, a combination of IL-1β and IFN-γ facilitates ER Ca^{2+} depletion mediated through inhibition of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2B), as well as production of NO in pancreatic β cells [56]. The activation of IRE1α by these cytokines is likely to be transient. The study by Brozzi et al showed IL-1β and IFN-γ gradually facilitates IRE1α activation and peaks at 16 h before declining [33]. This may explain why only activation of PERK-EIF2A was observed in this study where the incubation time was limited to 24 h. Finally, the IRE1α signaling pathway has been linked to cellular inflammatory response mechanisms through the JNK and NF-kB pathways, thereby possibly providing a positive feedback loop [57].

Other stressors closely linked to the pre-eclampsia may also act through the same

pathways, for example, high levels of maternal endothelin-1 (ET-1) or elevated plasma concentrations of homocysteine [58-60]. We have demonstrated ET-1 down-regulates MMP14 and 15 expression in first trimester trophoblast cells [61], and both ET-1 and homocysteine induce ER stress in trophoblast and other cell types [62, 63]. Therefore, the finding of ER stress–regulated *MMP2* expression may provide an additional mechanistic explanation for the actions of ET-1 and homocysteine in the development of pregnancy complications.

These results elucidated the co-existence of both transcriptional and translational regulation of MMP-2 by ATF4 and EIF2A, respectively, under ER stress. This finding is supported by the siRNA-mediated knockdown of *ATF4* gene and GSK2606414-mediated suppression of phosphorylation of EIF2A in restoration of *MMP2* transcript and protein levels, respectively (Figure 5E and F). Interestingly, ATF4 nuclear localization was blocked by GSK2606414 treatment under ER stress. Although the mechanism behind this failure is unknown, recent studies have revealed roles for post-translational modifications in determination of ATF4 protein stability, nuclear localization, and transcriptional activity [64]. Nevertheless, without nuclear translocation the inhibitory role of ATF4 in *MMP2* transcription is minimal, thereby facilitating both transcription and translation of MMP-2 in the presence of GSK2606414. A combination of both transcriptional and translational regulation of MMP-2 ensures no cells invade

into or towards an unfavorable environment.

Finally, these results are consistent with the study by Lian et al, showing that decidual ER stress is increased in pregnancies complicated by fetal growth restriction and pre-eclampsia via up-regulation of the PERK/EIF2A signaling mechanism [38]. We recognize that use of placental bed samples collected following delivery at term was a limitation in this study. Ideally, placental bed samples from the first trimester are the most appropriate, but these are impossible to obtain for ethical and technical reasons. The use of trophoblast cell lines is also not ideal, but primary trophoblast cells demonstrate high levels of ER stress induced during the isolation procedure (Yung, unpublished data). This stress would mask the low-grade ER stress induced by the treatment with pro-inflammatory cytokines and confound the experiments.

To conclude, although there may be other mechanistic links for the inhibition of trophoblast invasion by pro-inflammatory cytokines, this study provides the first evidence that ER stress plays a role through the PERK-EIF2A-ATF4 arm of the UPR pathway. The results further elucidate a potential pathophysiologic bridge across inflammation, ER stress, and sub-optimal trophoblast invasion in pregnancy, explaining why women with uterine or metabolic inflammation have an increased risk of developing pre-eclampsia. These new insights highlight for potential therapeutic

interventions aimed at alleviating ER stress at the fetal-maternal interface, facilitating trophoblast invasion and promoting normal placentation.

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C.L.L. acquired and analyzed data and wrote the manuscript. J.H.W.V., T.K.R., and B.B.van R. acquired and analyzed data. G.J.B. and H.W.Y. designed the study, analyzed the data and, wrote the manuscript.

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Figure legends

Figure 1. Endoplasmic reticulum (ER) stress is present in extravillous trophoblasts (EVTs) of placental bed samples from pre-eclamptic patients. Immunohistochemistry for the trophoblast marker CK7 and ER stress biomarker HSPA5/GRP78 was performed on serial sections from placental bed samples from both pre-eclamptic patients and normotensive control (Upper panel: 50x magnification, scale bar = 500 µm; Lower panel: 200x magnification, scale bar = 100 µm). Placental villous tissue was used as a positive control for HSPA5 immunostaining.

Figure 2. Endoplasmic reticulum (ER) stress reduces matrix metallopeptidase (MMP)-2 expression, secretion, and activity. JEG-3 cells were subjected to a dose-response study with tunicamycin at 0.31, 0.62, and 1.25 µg/mL for 24 h. **A:** Levels of P-EIF2A, EIF2A, ATF4, HSPA5, and GRP94 were measured by western blotting. **B:** MMP-2 expression and secretion were determined by western blot whereas activity was analyzed by gelatin gel zymography. **C:** Transcript levels of *MMP2* were quantified by quantitative reverse transcription-PCR. All data presented as mean ± SEM, N=3. ***P* < *0.01*.

Figure 3. Endoplasmic reticulum (ER) stress mediates down-regulation of matrix metallopeptidase (MMP)-2 activity and is associated with a reduction in

trophoblast invasiveness. HTR8/SVneo were treated with 0.078 μg/mL tunicamycin (Tm) or 125 nM thapsigargin (Tg) for 24 h. **A:** Levels of P-EIF2A, EIF2A, HSPA5, and GRP94 were determined by western blotting. **B:** Densitometry analysis of P-EIF2A, EIF2A, HSPA5, and GRP94. **C:** Levels of MMP-2 and secretion of MMP-2 and TIMP1 were determined by western blotting. MMP-2 activity was measured by gelatin gel zymography. **D:** Densitometry analysis of cellular, secreted, and active MMP2. **E:** Invasion of trophoblast cells was determined by trans-well invasion assay with SYTOX-green fluorescence staining. **F:** Quantification of the rate of invasion (% to control). All data above are expressed as mean ± SEM, N=3. *$P < 0.05$. **$P < 0.01$.

Figure 4. Pro-Inflammatory cytokines inhibit trophoblast MMP-2 matrix metallopeptidase (MMP)-2 levels, secretion, and activity, and also induce endoplasmic reticulum (ER) stress. JEG-3 cells were treated with interleukin (IL)-1β, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and all cytokines for 24 h. **A:** MMP2 levels and secretion were determined by western blotting. MMP-2 activity was analyzed by gelatin gel zymography. **B:** Densitometry of the cellular, secreted, and active MMP-2. Data are presented as mean ± SEM. N=4. *$P < 0.05$. **C:** Levels of ER stress biomarkers, P-EIF2A, EIF2A, and HSPA5 were measured by western blotting. **D:** Densitometry analysis of the P-EIF2A, EIF2A, and HSPA5 and data were presented as mean ± SEM, N=4 to 9. *$P < 0.05$; **$P < 0.01$. **E:** Regression analysis showing the correlation between

MMP-2 level, secretion, and activity with P-EIF2A level.

Figure 5. Endoplasmic reticulum (ER) stress–mediated suppression of matrix metallopeptidase (MMP)-2 expression is via the EIF2A-ATF4 pathway. JEG-3 cells were treated with *ATF4* siRNA for 48 h followed by 100 nM of thapsigargin (Tg) treatment for 24 h. **A:** Regression analysis showing the correlation between *MMP2* expression and secretion with ATF4 level. **B:** Quantitative reverse transcription-PCR reveals up-regulation of *MMP2* mRNA in *siATF4*-transfected cells with or without Tg treatment. *$P < 0.05$. **C:** Levels of P-EIF2A, EIF2A, ATF4, cellular MMP-2, and secreted MMP-2 in *siATF4*-transfected cells were determined by western blotting. MMP-2 activity was determined by gelatin gel zymography. **D:** Densitometry of the level of ATF4, cellular, secreted, and active MMP-2. All data are presented as mean ± SEM, N=3 to 5 *$P < 0.05$; ***$P < 0.001$. **E:** MMP-2 protein under ER stress was measured in the presence or absence of PERK inhibitor, GSK2606414. JEG3 cells were treated with ER stress inducer and/or GSK2606414 (100 nM) before Western blotting for MMP-2, ATF4, P-EIF2A, and EIF2A. **F:** Immunofluorescence was used to show the localization of ATF4 protein under ER stress in the presence or absence of GSK2606414. Images were taken at 200X magnification. Scale bar = 50 μm.

Table 1. Clinical Characteristics of normotensive and early-onset preeclamptic patients.

Characteristics	**Normotensive Control (n=9)**	**Early-onset Preeclampsia (n=7)**	***P-value***
Maternal Age (year)	33 ± 1.6	28.9 ± 1.8	0.148
BMI	24.2 ± 1.7	27.8 ± 2.2	0.204
Blood Pressure (Systolic)	122.6 ± 2.4	187.9 ± 4.2	*0.001*
Blood Pressure (Diastolic)	76.7 ± 1.9	113 ± 2.5	*0.001*
Gestational Age (day)	274.8 ± 0.8	208.4 ± 5.1	*0.001*
Birth Weight (gram)	3635.2 ± 111.4	1037.9 ± 115.3	*0.001*

Figure 1

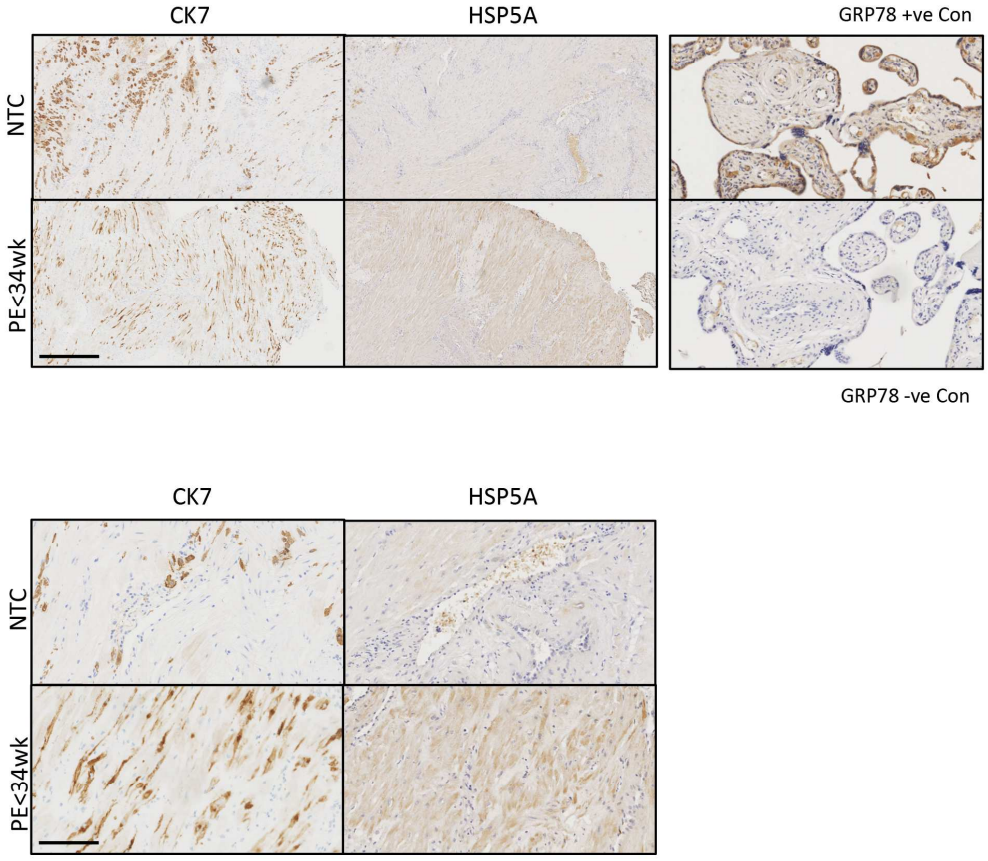
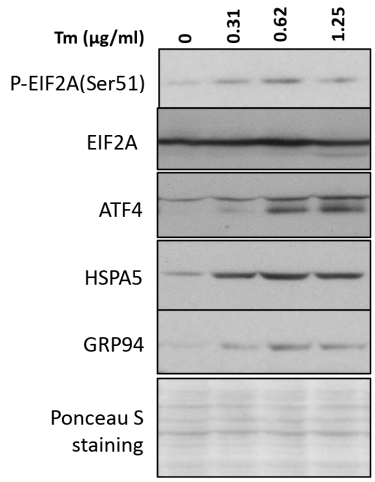
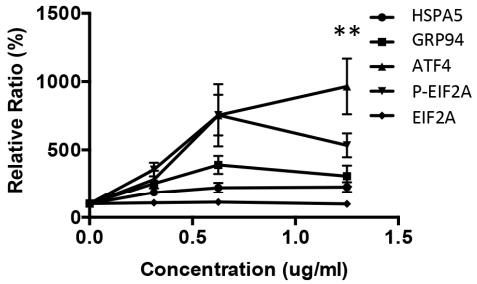

CK7
HSP5A
GRP78 +ve Con
NTC
PE<34wk
GRP78 -ve Con
CK7
HSP5A
NTC
PE<34wk

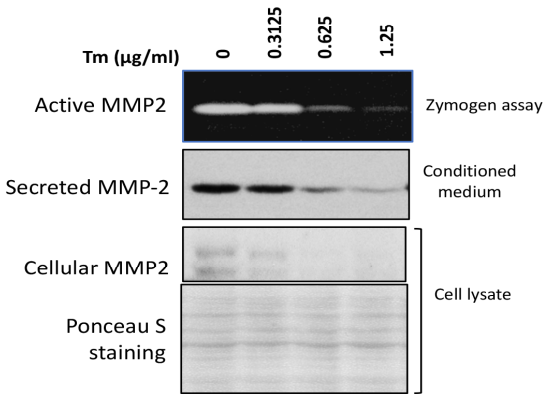
Figure 2

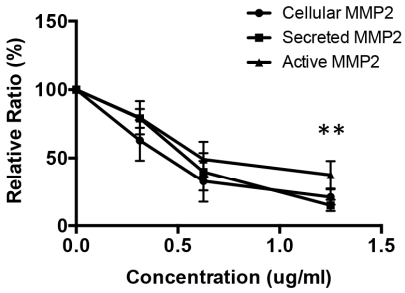
A


Tm (µg/ml)
0
0.31
0.62
1.25
P-EIF2A(Ser51)
EIF2A
ATF4
HSPA5
GRP94
Ponceau S staining


Relative Ratio (%)
1500
1000
500
0
0.0
0.5
1.0
1.5
Concentration (ug/ml)
**
HSPA5
GRP94
ATF4
P-EIF2A
EIF2A

B


Tm (µg/ml)
0
0.3125
0.625
1.25
Active MMP2
Zymogen assay
Secreted MMP-2
Conditioned medium
Cellular MMP2
Cell lysate
Ponceau S staining


Relative Ratio (%)
150
100
50
0
0.0
0.5
1.0
1.5
Concentration (ug/ml)
**
Cellular MMP2
Secreted MMP2
Active MMP2

C

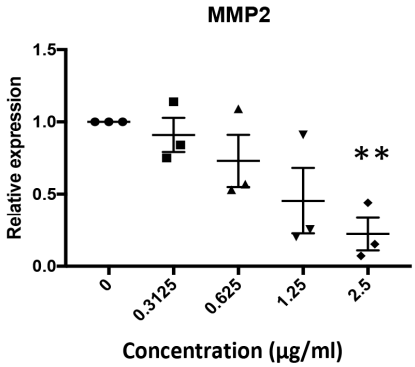

MMP2
Relative expression
1.5
1.0
0.5
0.0
0
0.3125
0.625
1.25
2.5
**
Concentration (µg/ml)

Figure 3

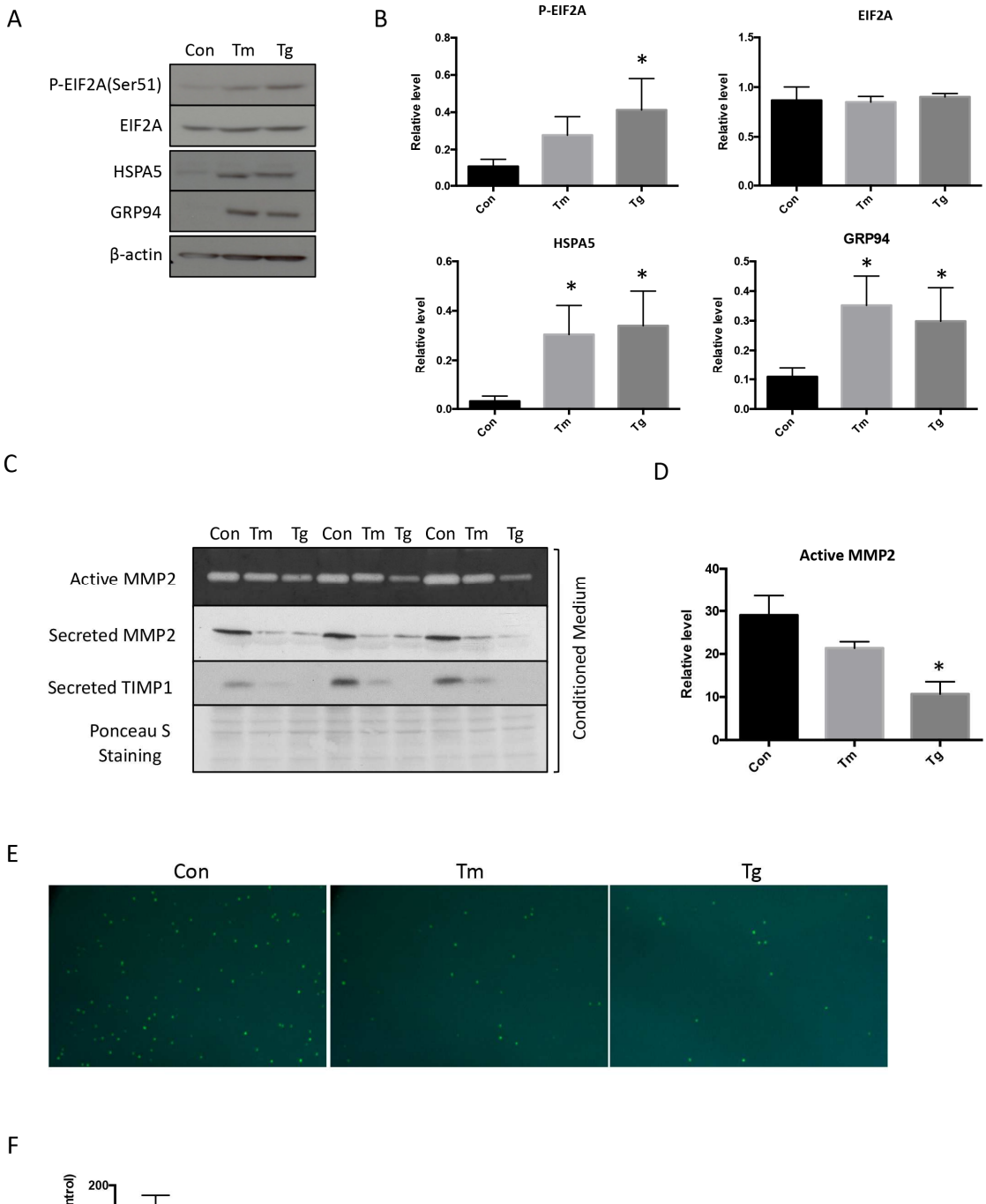

A
Con Tm Tg
P-EIF2A(Ser51)
EIF2A
HSPA5
GRP94
β-actin
B
P-EIF2A
EIF2A
HSPA5
GRP94
Relative level
Con
Tm
Tg
C
Con Tm Tg Con Tm Tg Con Tm Tg
Active MMP2
Secreted MMP2
Secreted TIMP1
Ponceau S Staining
Conditioned Medium
D
Active MMP2
Relative level
E
Con
Tm
Tg
F
Rate of invasion (% to control)

Figure 4

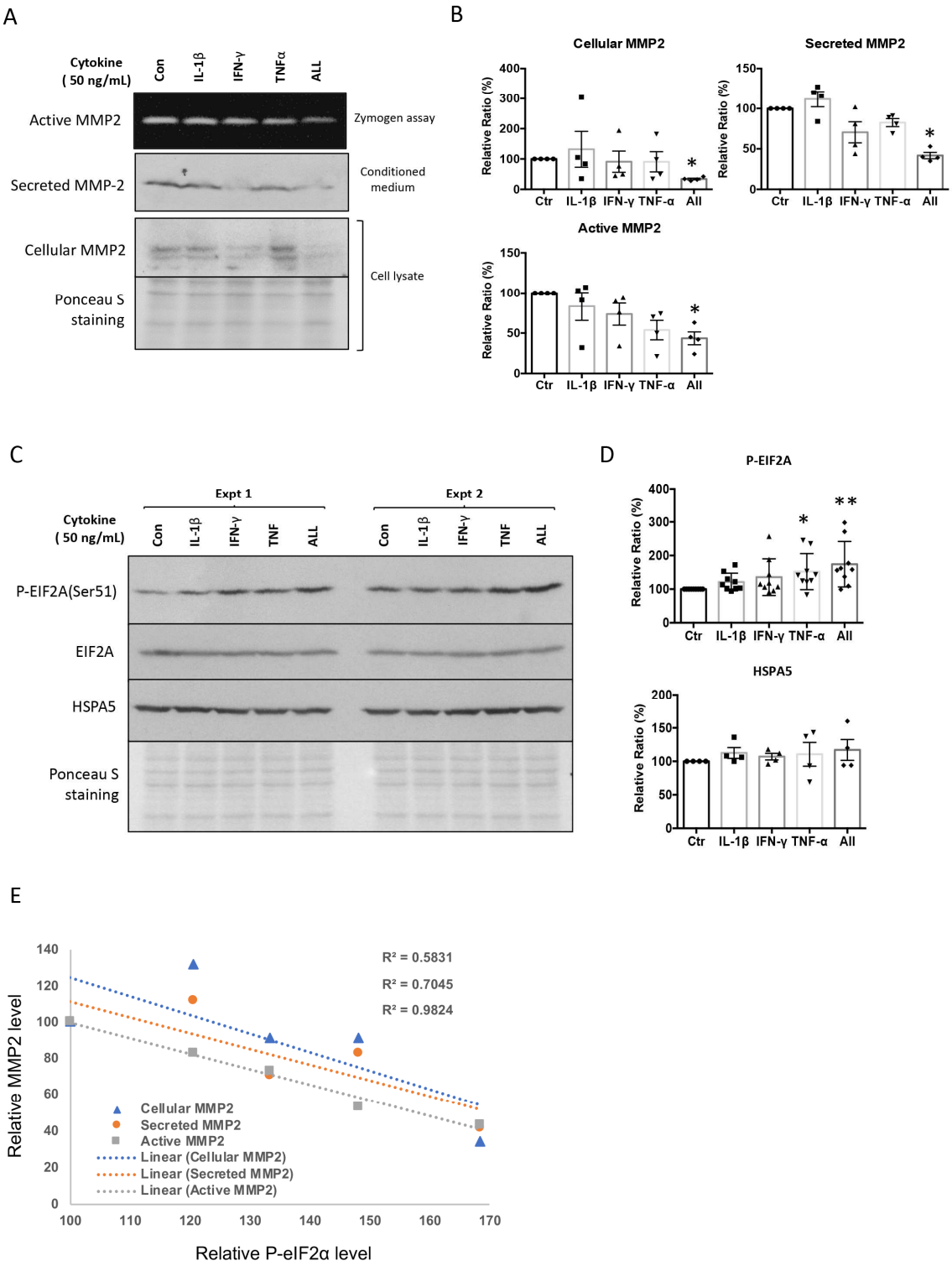
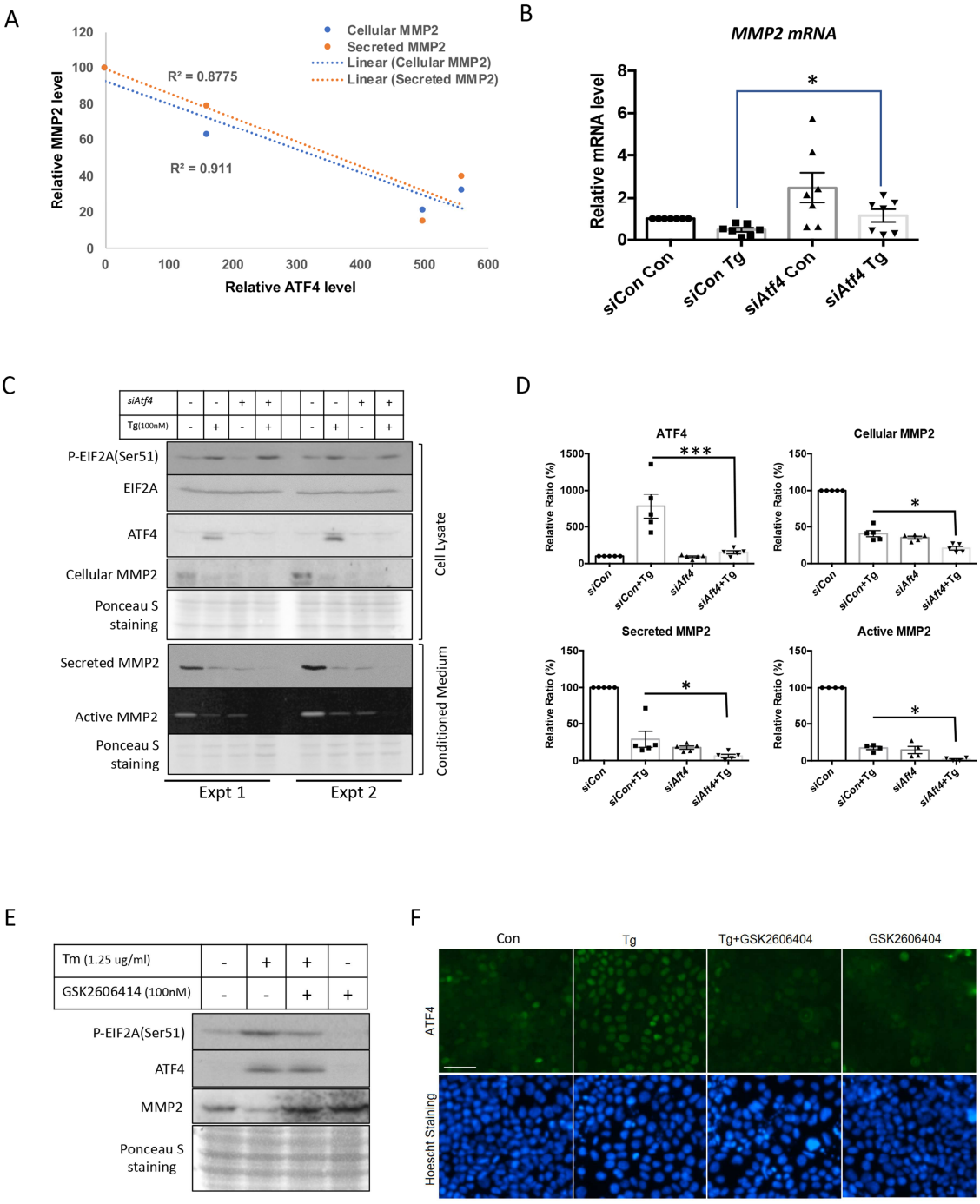

A
Cytokine (50 ng/mL)
Con
IL-1β
IFN-γ
TNFα
ALL
Active MMP2
Zymogen assay
Secreted MMP-2
Conditioned medium
Cellular MMP2
Ponceau S staining
Cell lysate
B
Cellular MMP2
Secreted MMP2
Active MMP2
Relative Ratio (%)
Ctr
IL-1β
IFN-γ
TNF-α
All
C
Expt 1
Expt 2
Cytokine (50 ng/mL)
Con
IL-1β
IFN-γ
TNF
ALL
P-EIF2A(Ser51)
EIF2A
HSPA5
Ponceau S staining
D
P-EIF2A
HSPA5
Relative Ratio (%)
Ctr
IL-1β
IFN-γ
TNF-α
All
E
Relative MMP2 level
Relative P-eIF2α level
R² = 0.5831
R² = 0.7045
R² = 0.9824
Cellular MMP2
Secreted MMP2
Active MMP2
Linear (Cellular MMP2)
Linear (Secreted MMP2)
Linear (Active MMP2)

Figure 5


A
Cellular MMP2
Secreted MMP2
Linear (Cellular MMP2)
Linear (Secreted MMP2)
R² = 0.8775
R² = 0.911
Relative MMP2 level
Relative ATF4 level
B
MMP2 mRNA
Relative mRNA level
siCon Con
siCon Tg
siAtf4 Con
siAtf4 Tg
C
siAtf4
Tg(100nM)
P-EIF2A(Ser51)
EIF2A
ATF4
Cellular MMP2
Ponceau S staining
Cell Lysate
Secreted MMP2
Active MMP2
Ponceau S staining
Conditioned Medium
Expt 1
Expt 2
D
ATF4
Cellular MMP2
Secreted MMP2
Active MMP2
Relative Ratio (%)
siCon
siCon+Tg
siAtf4
siAtf4+Tg
E
Tm (1.25 ug/ml)
GSK2606414 (100nM)
P-EIF2A(Ser51)
ATF4
MMP2
Ponceau S staining
F
Con
Tg
Tg+GSK2606404
GSK2606404
ATF4
Hoescht Staining