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ORIGINAL ARTICLE *Early pregnancy*

Endothelin-1 down-regulates matrix metalloproteinase 14 and 15 expression in human first trimester trophoblasts via endothelin receptor type B

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STUDY QUESTION: Does endothelin-1 (ET-1) regulate matrix metalloproteinase (MMP) 14 and 15 production and invasion of human first trimester trophoblasts?

SUMMARY ANSWER: ET-1 in pathophysiological concentrations down-regulates MMP14 and MMP15 expression via endothelin receptor (ETR) type B and decreases trophoblast migration and invasion.

WHAT IS KNOWN ALREADY: MMP14 and MMP15 are involved in trophoblast invasion. Impairment of invasion has been linked to pregnancy complications such as pre-eclampsia (PE). ET-1 is up-regulated in PE.

STUDY DESIGN, SIZE, DURATION: *In vitro* study using primary human trophoblasts from 50 first trimester placentas (gestational week 7–12).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Trophoblasts were cultured in the absence or presence of 10–100 nM ET-1. MMP14 and MMP15 mRNA and protein were quantified by RT-qPCR and Western blotting, respectively. Selective antagonists for ETRA (BQ-123) or ETRB (BQ-788) were used to identify ETR subtypes involved. Functional ET-1 effects were tested in first trimester chorionic villous explants and transwell invasion assays. The roles of tumor necrosis factor (TNF)-α (25 ng/ml) and oxygen (1%) in ET-1 regulation of MMP14 and 15 expression were assessed by Western blotting.

MAIN RESULTS AND THE ROLE OF CHANCE: ET-1 down-regulated MMP14 and MMP15 mRNA (−21% and −26%, respectively, $P < 0.05$) and protein levels (−18% and −22%, respectively, $P < 0.05$). This effect was mediated via ETRB. ET-1 decreased trophoblast outgrowth in placental explants (−24%, $P < 0.05$) and trophoblast invasion (−26%, $P \leq 0.01$). TNF-α enhanced ET-1 mediated MMP15 down-regulation (by 10%, $P < 0.05$), whereas hypoxia abolished the effect of ET-1 on both MMPs.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Only primary trophoblasts were used in this study. Since trophoblast yield from first trimester placental material is limited, further aspects of MMP14 and 15 regulation could not be characterized. Other anti-invasive factors may

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be altered by ET-1 in trophoblasts and, thus, contribute to the reduced invasion, but have not been investigated. Oxygen levels similar to those found in the decidua (5–8% O_2) were not analyzed in this study.

WIDER IMPLICATIONS OF THE FINDINGS: ET-1 modifies placental function already during the first trimester of pregnancy, the time-window when the placental changes implicated in PE occur. Thus, our results improve the understanding of the placental mechanisms underlying trophoblast invasion and PE.

STUDY FUNDING/COMPETING INTEREST(S): The study was funded by the Oesterreichische Nationalbank (Anniversary Fund, project number: 14796) and the Herzfelder'sche Familienstiftung (to J.P.; number: 00685). AMM received funding from the Austrian Science Fund FWF (W1241) and the Medical University Graz through the PhD Program Molecular Fundamentals of Inflammation (DK-MOLIN). The authors have no conflict of interest.

Key words: endothelin-1 / MMPs / pre-eclampsia / invasion / trophoblast / inflammation / hypoxia

Introduction

During the first trimester of pregnancy trophoblast differentiates along two pathways: (i) villous trophoblast (VT), and (ii) extravillous trophoblast (EVT). VT are proliferative cells which can fuse to form the syncytiotrophoblast, a multinucleated layer involved in nutrient and oxygen transportation and hormone production. EVT are invasive cells which migrate from the tip of the placental villi and invade the uterine wall (Knöfler, 2010). EVT also reach and remodel the spiral arteries, transforming them into low resistance vessels, a process necessary to allow an adequate blood supply to the fetus (Kaufmann *et al.*, 2003).

EVT migration and invasion, as well as spiral artery remodeling, require degradation of the extracellular matrix (ECM). For that purpose, trophoblast cells express a variety of proteases, including serine proteases, cathepsins and matrix metalloproteinases (MMPs) (Ghaffari-Tabrizi-Wizsy *et al.*, 2014). The latter are a family of 24 zinc-dependent endopeptidases capable of degrading virtually all the components of the ECM. MMPs are secreted as zymogens containing a pro-domain, and their activity can be regulated at several levels, e.g. transcription, activation and inhibition by tissue inhibitors of MMPs (TIMPs) (Nagase *et al.*, 2006). A subfamily of MMPs are membrane-type MMPs (MT-MMPs), containing a membrane insert. These MT-MMPs are important in many aspects of physiology and pathophysiology of pregnancy (Majali-Martinez *et al.*, 2016). We and others have shown that in primary first trimester trophoblasts only two members of the MT-MMP subfamily, MMP14 and MMP15 are highly expressed (Hiden *et al.*, 2013), with MMP15 being specifically expressed in EVT (Pollheimer *et al.*, 2014). Moreover, MMP14 and MMP15 are the main activators of MMP2 (Itoh, 2015), a secreted MMP also known to play a pivotal role in trophoblast invasion (Xu *et al.*, 2000). Therefore, MMP14 and 15 are considered key players in trophoblast invasion.

Both, MMP expression and trophoblast invasion have to be tightly regulated to ensure a successful pregnancy. Indeed, pre-eclampsia (PE) has been linked to an impairment of trophoblast invasion already in the first trimester (Kaufmann *et al.*, 2003). PE occurs in 2–8% of pregnancies and is one of the major causes of maternal morbidity and mortality. Thus, current efforts aim to identify biomarkers that would allow improved management of pregnancies at risk for PE (Rodriguez *et al.*, 2016).

Endothelin-1 (ET-1) has gained considerable attention in PE research (George and Granger, 2012). It acts through its two G protein-coupled receptors, ETR type A (ETRA) and type B (ETRB) (Luscher and Barton, 2000). ET-1 regulates the vascular tone but also modulates proliferation, migration and invasion of several cell types (Liu *et al.*, 2012; Chiriboga *et al.*, 2016). In PE, ET-1 is up-regulated due to the endothelial cell dysfunction characteristic of this condition (Lamarca, 2012). Interestingly, higher maternal ET-1 levels during the first trimester have been related to an increased risk of developing PE later in pregnancy (Shaarawy and Abdel-Magid, 2000). Moreover, first trimester trophoblasts secrete ET-1 and express both ETRA and ETRB (Cervar and Desoye, 1998).

Given the strong role of ET-1, MMP14 and MMP15 in modulating trophoblast invasion, we hypothesized that ET-1 regulates MMP14 and MMP15 expression in human primary first trimester trophoblasts entailing functional consequences for trophoblast invasion. The risk of PE is higher in pro-inflammatory conditions such as obesity (Jeyabalan, 2013), and may change oxygen tension in the intervillous space through delayed or inadequate opening of the spiral arteries (Ouyang *et al.*, 2009). Therefore, we tested whether a pro-inflammatory cytokine, tumor necrosis factor (TNF)-α or hypoxia further modulate the ET-1 effect on MMP14 and MMP15.

Material and Methods

First trimester trophoblast isolation

The study was approved by the institutional review board and ethical committee of the Medical University of Graz (24-129 ex 11/12) and the Medical University of Vienna (084/2009). Signed informed consent was obtained from the pregnant women. Trophoblasts were isolated from 50 first trimester placentas (gestational week (GW) 7–12) after pregnancy termination for psychosocial reasons as described elsewhere (Blaschitz *et al.*, 2000). GW was determined by ultrasound measurement of crown-rump length, and only placentas from similar gestational ages, i.e. early (GW 7 + 8), mid (GW 9 + 10) or late (GW 11 + 12) first trimester, were pooled before trophoblast isolation when required. Briefly, placental villi were digested with Dispase/DNAse and Trypsin (Gibco, Invitrogen, Carlsbad, CA, USA). After Percoll (Gibco) gradient centrifugation, cells were incubated with magnetic beads conjugated with anti-CD90 and anti-CD45 (Dako, Glostrup, DK) antibodies for 30 min and placed on a DynaMag-15 magnet (Thermo Scientific, Rockford, IL, USA) to remove fibroblasts and common leukocyte antigen expressing cells. The cells obtained constituted a mixture of VT and EVT. Functional activity and purity of trophoblast isolations were assessed by secretion of human chorionic

gonadotropin (hCG) (Dade Behring, Deerfield, IL, USA) and immunocytochemical staining for cytokeratin 7 (Dako, 1:750) and human leukocyte antigen (HLA)-G (BD-Biosciences, Bedford, MA, USA, 1:500), respectively. Only functionally active preparations with a purity ≥95% were used.

Cell culture

Isolated trophoblasts were seeded in gelatin pre-coated plates and maintained for 2 days in Keratinocyte medium (KCM, Gibco) with penicillin/streptomycin (Gibco), KCM supplements (Gibco) and 10% (v/v) fetal calf serum (FCS, Thermo Scientific) in a humidified incubator at 37°C, 5% CO_2 in air. Prior to treatments, the cells were cultured under low serum conditions (2% (v/v) FCS) for 24 h. Thereafter, the cells were incubated in the absence (control) or presence of 10 nM or 100 nM ET-1 (Sigma Aldrich, St. Louis, MO, USA) for 24 h. The ETR subtype involved in mediating ET-1 effects was determined by pre-incubating trophoblasts with selective ETR antagonists for 2 h prior to the addition of 100 nM ET-1: BQ-123 (Tocris, Bristol, UK, 1.4 and 11.2 nM) for ETRA and BQ-788 (Tocris, 1.2 and 9.6 nM) for ETRB. Because late first trimester (GW 11 + 12) placentas gave highest trophoblast yields, these isolations were used in this set of experiments. Trophoblasts were also treated with TNF-α (Sigma Aldrich, 25 ng/ml) either alone or in combination with 100 nM ET-1, or incubated in the absence or presence of 100 nM ET-1 under three different oxygen tensions (1%, 2.5% and 20% O_2) for 24 h.

RNA isolation and RT-qPCR analysis

Total trophoblast RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, DE). RNA (250 ng) was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Life Technologies-Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. *MMP14* and *MMP15* expression was determined by Real-time quantitative PCR (RT-qPCR) using FAM-labeled TaqMan gene expression assays (Life Technologies, *MMP14*: Hs01037003_g1; *MMP15*: Hs00233997_m1), TaqMan universal PCR master mix (Life Technologies) and the CFX96 real-time PCR detection system (BioRad Laboratories, Hercules, CA, USA). Ct values were automatically generated by the BioRad CFX Manager 3.0 software and relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method, with β-actin (Life Technologies, *ACTB*: Hs01060665_g1) as the reference gene.

Protein quantification by Western blotting and ELISA

Lysates of total cell proteins were prepared in RIPA buffer (Sigma Aldrich) containing protease inhibitors (Roche, Mannheim, DE), mixed with Laemmli buffer (Sigma Aldrich) and denatured at 96°C for 5 min. Samples were loaded onto 10% (v/v) SDS-PAGE gels (Bio-Rad Laboratories), resolved at 140 V for 1 h and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Non-specific binding sites were blocked for 1 h with 5% (w/v) non-fat dry milk (Bio-Rad Laboratories) in tris-buffered saline (TBS) + 0.1% (v/v) Tween 20 (Sigma Aldrich). After blocking, the membranes were incubated with antibodies against MMP14 (Millipore, Billerica, MA, USA, 1:1500), MMP15 (Millipore, 1:500), HLA-G (BD-Biosciences, 1:1000), GAPDH (Novus, Littleton, CO, USA, 1:20 000) or β-actin (Abcam, Cambridge, MA, USA, 1:25 000) overnight at 4°C. Blots were subsequently washed and incubated with the appropriate HRP-conjugated secondary antibody (Bio-Rad Laboratories, 1:2500 for MMP14, 1:1000 for MMP15; 1:2000 for HLA-G, 1:6000 for GAPDH and 1:25 000 for β-actin). Immunolabeling was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized with the Chemidoc XRS software (Bio-Rad Laboratories). Band density was quantified using the Alpha Digidoc software (Alpha Innotech Corp, Innsbruck, AUT). GAPDH or β-actin were used as loading control.

Cell supernatants from controls under 1%, 2.5% and 20% O_2 (5% CO_2 balance N_2) were collected and concentrated through column centrifugation (Sartorius Stedim, Goettingen, DE) to a final volume of 500 µl. ET-1 concentrations were determined by ELISA according to manufacturer's instructions (R&D, Abingdon, UK) and normalized to total protein concentration in the supernatants.

Explants of first trimester chorionic villi

First trimester placental explants were prepared following established protocols (Vicovac *et al.*, 1995). Briefly, small pieces of tissue (2–4 mm^2) from the periphery were dissected under the microscope and maintained in DMEM/Ham's F12 medium (Gibco) without serum supplementation. ECM was prepared by mixing collagen I (Corning, Bedford, MA) with 10× DMEM (Gibco, 1:10, v/v) and 7.5% sodium bicarbonate (Sigma, 1:5, v/v) followed by incubation at 37°C for gel formation. The dissected placental tissue was then placed on the top of the gel and incubated for 4 h for anchorage. Medium without (control) or with 100 nM ET-1 was subsequently added for 24 h. Trophoblast outgrowth was measured as the distance from the villous margin to the outer edge of the migrating cell layer.

Transwell invasion assay

Trophoblast invasion was determined using 12 mm Transwell inserts with 12 µm pores (Millipore) pre-coated with 1 mg/ml fibronectin (Millipore). Inserts were placed into a 24-well plate containing 400 µl DMEM/Ham's F-12 medium supplemented with 10% (v/v) FCS. After isolation, trophoblasts were re-suspended in 300 µl medium without FCS in the absence (control) or presence of 100 nM ET-1, and seeded in the upper chamber. After 48 h, the inserts were fixed with ice-cold methanol and non-invading cells were removed with a cotton swab. Invading cells were stained with DAPI and five different fields were counted under the microscope with a magnification of 100×.

Statistics

Statistical analyses were performed using Graphpad (version 6.5). The results presented are representative for at least three independent experiments. After testing for normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk), statistical significance was determined by *t*-test or ANOVA as appropriate, and $P < 0.05$ was considered statistical significant.

Results

ET-1 down-regulates MMP14 and 15 mRNA and protein levels

ET-1 had a dose-dependent effect on MMP14 and 15 mRNA, with 100 nM ET-1 down-regulating both MMP14 (−21%; $P \leq 0.01$) and MMP15 (−26%; $P \leq 0.001$) expression in primary trophoblasts (GW 7 + 8, Fig. 1A).

For protein analysis, samples were divided into early (GW 7 + 8) and mid (GW 9 + 10) first trimester trophoblasts. For MMP14, only the active form (act-MMP14) was observed, whereas for MMP15, pro-MMP15 and active-MMP15 (act-MMP15) were detected at the expected molecular weights (Fig. 1B). In concordance with the mRNA results, ET-1 dose-dependently reduced act-MMP14 (−18%; $P < 0.05$)

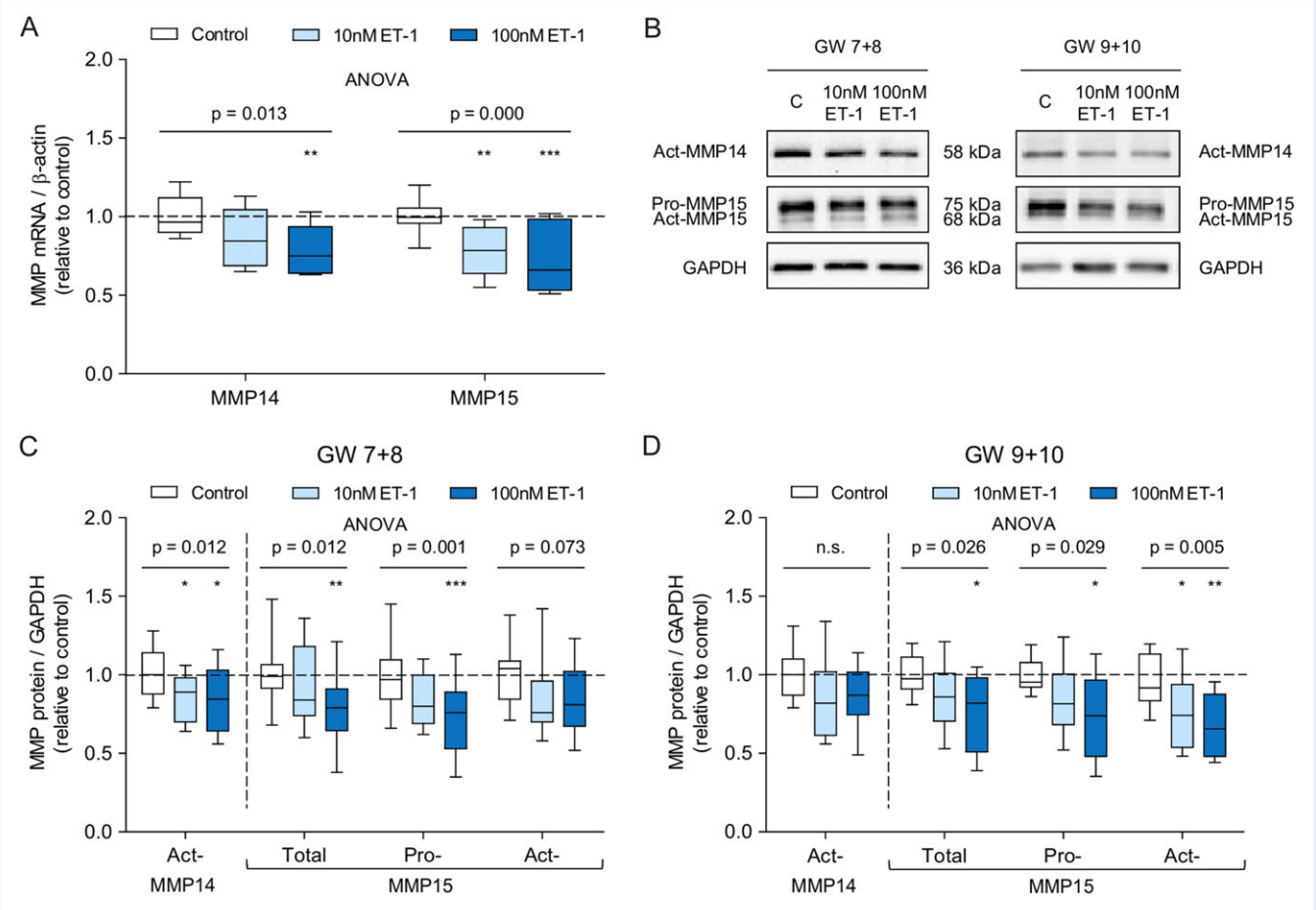

Figure 1 Endothelin-1 (ET-1) down-regulates matrix metalloproteinase (MMP) 14 and MMP15 mRNA and protein levels in primary trophoblast: (**A**) MMP14 and MMP15 expression was determined by RT-qPCR (GW 7 + 8, $n = 5$); (**B–D**) For Western blot analysis, trophoblast isolations were separated according to gestational week (GW 7 + 8, $n = 9$; and 9 + 10, $n = 4$); MMP14 (active form, act-MMP14) and MMP15 (pro-MMP15, active: act-MMP15, total-MMP15: pro + act-MMP15) were detected; Results were normalized to β-act expression or GAPDH protein levels and calculated as fold change relative to the controls, arbitrarily set to 1; Data are representative for at least three experiments; n.s., non-significant; $^{*}P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ vs control.

and total-MMP15 (sum of pro- and act-MMP15; −22%; $P \leq 0.01$) protein levels in primary trophoblasts from GW 7 + 8. This was also true for pro- and act-MMP15 when analyzed separately (Fig. 1C). The ET-1 effect on MMP15 (total, pro- and active forms) was maintained in trophoblasts from GW 9 + 10, whereas MMP14 down-regulation showed a similar tendency without reaching statistical significance (Fig. 1D).

MMP14 and 15 down-regulation is mediated via ETRB

ETR involvement in MMP14 and MMP15 down-regulation was determined in late first trimester primary trophoblasts (GW 11 + 12) using two specific antagonists for ETRA and ETRB. We firstly confirmed the effect of ET-1 on MMP14 and MMP15 down-regulation (Fig. 2, −21%; $P < 0.05$ and −22%; $P \leq 0.01$; respectively). Pre-incubation with ETRA antagonist BQ-123 enhanced the effect of ET-1 on act-MMP14 and total-MMP15 down-regulation by 16% and 15%, respectively (Fig. 2A–C, 100 nM ET-1 vs 11.2 nM BQ-123 + 100 nM ET-1, $P < 0.05$), whereas blocking ETRB with BQ-788 abolished the effect of ET-1 on MMP down-regulation (Fig. 2D–F). Similar results were obtained when pro- and act-MMP15 were analyzed separately (Supplementary data, Fig. S1). Altogether, these results indicate that MMP down-regulation is mediated via ETRB.

ET-1 hinders trophoblast migration and invasion

To test whether ET-1 impairs trophoblast function, trophoblast migration and invasion were determined. First trimester chorionic villi were cultured in the absence (Fig. 3A) or presence (Fig. 3B) of ET-1 and trophoblast outgrowth, i.e. migration from the villous margin, was determined (arrows, Fig. 3A and B). After 24 h, 100 nM ET-1 decreased trophoblast outgrowth by 24% (Fig. 3C; $P \leq 0.01$). Since trophoblast outgrowth reflects trophoblast migration, we additionally performed transwell invasion assays (Fig. 3D). In concordance with what we observed in the chorionic villi, 100 nM ET-1 decreased invasion of isolated trophoblasts (−26%; $P \leq 0.01$).

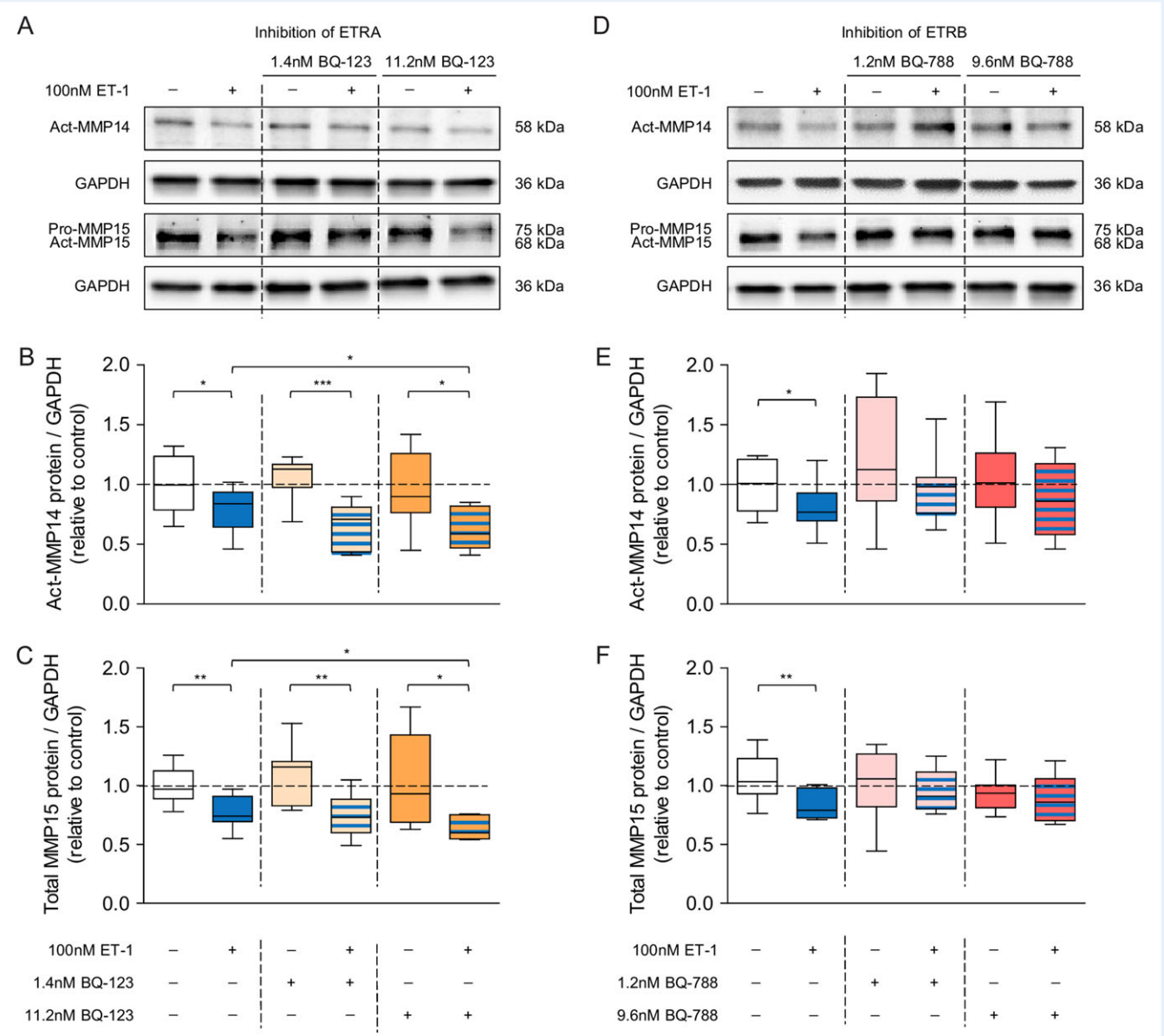

Figure 2 MMP14 and MMP15 down-regulation is mediated via endothelin receptor type B (ETRB): Primary trophoblasts (GW 11 + 12, $n = 7$) were pre-incubated with two selective ETR antagonists (**A–C**, BQ-123 for ETRA; **D**–**F**, BQ-788 for ETRB) for 2 h prior to the treatment with 100 nM ET-1; MMP14 (active form, act-MMP14) and MMP15 (pro-MMP15, act: active-MMP15, total-MMP15: pro + act-MMP15) protein levels were determined by Western blotting; Results were normalized to GAPDH protein levels and calculated as fold change relative to the controls, arbitrarily set to 1; Data are representative for at least four experiments; *$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

TNF-α enhances ET-1 mediated MMP15 down-regulation

TNF-α was used to assess whether a pro-inflammatory environment modulates the effect of ET-1 on MMP regulation. Alone, TNF-α had no effect on act-MMP14 or total-MMP15 protein levels when compared to the controls (Fig. 4). When administered together with ET-1, TNF-α did not alter the effect of ET-1 on act-MMP14 down-regulation. In contrast, TNF-α increased the effect of ET-1 on total-MMP15 down-regulation by 10% (Fig. 4, ET-1 vs ET-1 + TNF-α; $P < 0.05$). Similar results were obtained when pro- and act-MMP15 were analyzed separately (Supplementary data, Fig. S2).

Low oxygen tension abolishes the effect of ET-1 on MMP14 and 15 down-regulation

To determine whether hypoxia (1% O_2) modulates MMP14 and 15 levels and their regulation by ET-1, two different control conditions were used: (i) 2.5% O_2 mimicking first trimester placental oxygen levels (Tuuli *et al.*, 2011) and, (ii) 20% O_2 since cell isolation was performed under this condition.

When compared to 20% O_2, primary trophoblasts cultured under 1% O_2 had decreased act-MMP14 (−40%; $P \leq 0.01$) and pro-MMP15 (−45%; $P \leq 0.001$) protein levels (Fig. 5A–C). No differences were observed between 1% O_2 and 2.5% O_2.

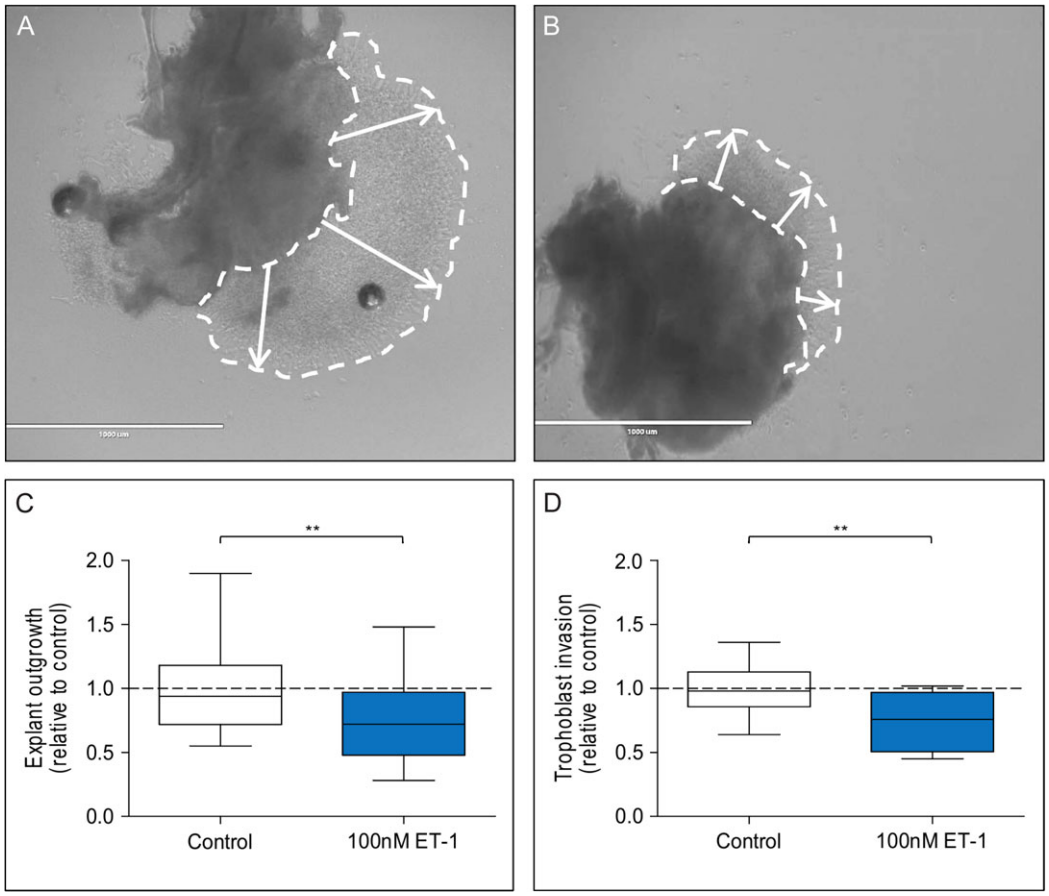

Figure 3 ET-1 hinders trophoblast migration and invasion: (**A–C**) Trophoblast migration was measured as trophoblast outgrowth (arrows, A and B) from chorionic villi (GW 7–11, $n = 4$) in the absence (control) or presence of 100 nM ET-1 for 24 h; (**D**) Trophoblast invasion was determined in transwell assays; For both assays results were calculated relative to the controls, arbitrarily set to 1; Data are representative for at least four experiments; $^{**}P \leq 0.01$.

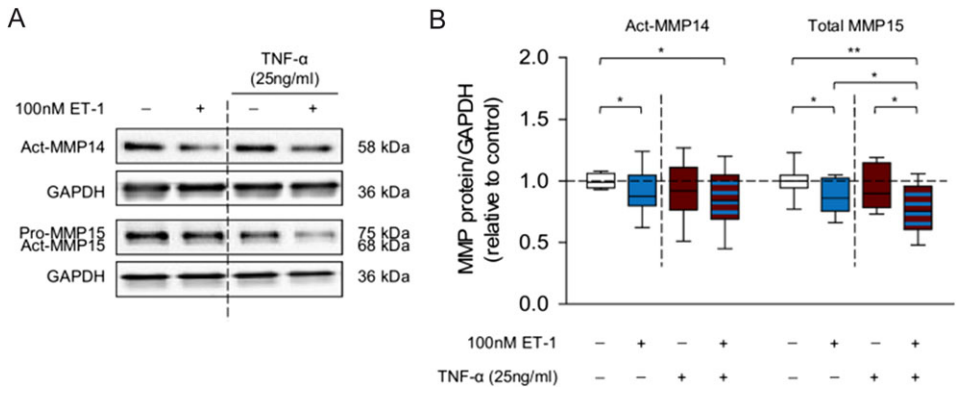

Figure 4 Tumor necrosis factor (TNF)-α enhances the effect of ET-1 on MMP15 down-regulation: Primary trophoblasts (GW 7–11, $n = 5$) were incubated in the absence (control) or presence of TNF-α (25 ng/ml) and 100 nM ET-1 either singly or in combination for 24 h; (**A**) MMP14 (active form, act-MMP14) and MMP15 (pro-MMP15, act-MMP15, total-MMP15: pro + act-MMP15) protein levels were determined by Western blotting; (**B**) Results were normalized to GAPDH protein levels and calculated as fold change relative to the controls, arbitrarily set to 1; Data are representative for at least four experiments; $^{*}P < 0.05$; $^{**}P \leq 0.01$.

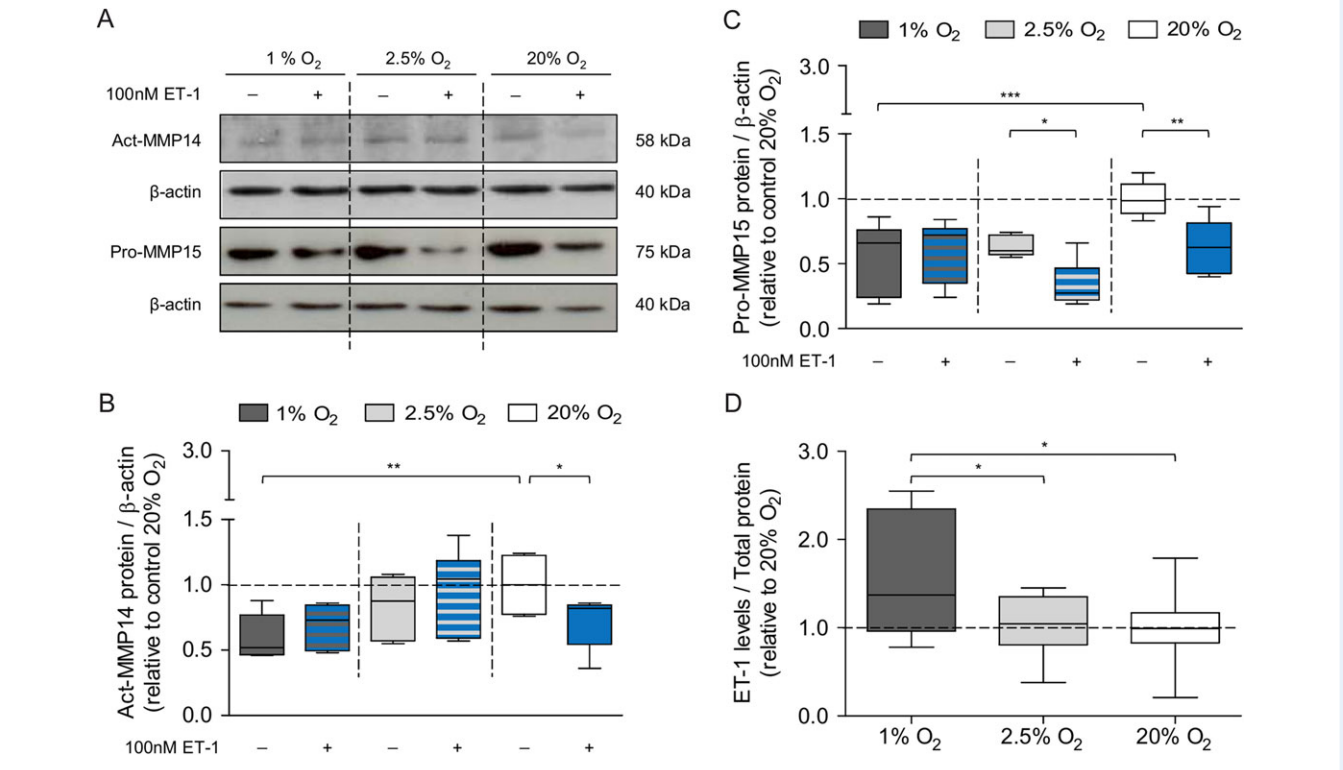

Figure 5 Low oxygen tension modulates the effect of ET-1 on MMP14 and MMP15 down-regulation: Primary trophoblasts (GW 7–11, $n = 12$) were incubated under three different O_2 tensions (1% O_2, 2.5% O_2 and 20% O_2) in the absence (control) or presence of 100 nM ET-1 for 24 h; (**A–C**) MMP14 (active form, act-MMP14) and MMP15 (pro-MMP15) protein levels were determined by Western blotting; (**D**) ET-1 levels were measured in the supernatants from the controls by ELISA; Results were normalized to β-actin protein levels (A and B) or total protein (D) and calculated as fold change relative to the controls (20% O_2), arbitrarily set to 1; Data are representative for at least four experiments; $^*P < 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

The effect of ET-1 on act-MMP14 and pro-MMP15 down-regulation was abolished under 1% O_2 (Fig. 5A–C). Interestingly, under 2.5% O_2 the effect of ET-1 on MMP14 down-regulation was also inhibited, whereas MMP15 down-regulation was increased (−53% under 2.5% O_2 vs −37% under 20% O_2, Fig. 5A and C).

To test whether these observations correlate with differences in endogenous ET-1 production, ET-1 levels were measured in the supernatants of primary trophoblasts not supplemented with ET-1 (Fig. 5D). ET-1 secretion was significantly increased under 1% O_2 (+55 and +50% vs 2.5% and 20% O_2, respectively, $P < 0.05$).

Discussion

Despite the increasing number of studies addressing the human placenta during the first trimester of pregnancy, the molecular mechanisms regulating trophoblast invasion are still not completely understood. We therefore assessed the role of ET-1 on MMP14 and 15 expression, two key proteases involved in trophoblast invasion (Hiden *et al.*, 2013; Pollheimer *et al.*, 2014). Our key findings were that ET-1 reduced MMP14 and 15 protein levels via ETRB in primary trophoblasts, and that this effect was paralleled by a decrease in trophoblast invasion.

ET-1 has been described to regulate MMPs in several cell types. However, these data refer mainly to MMP2 and 9 (Yao *et al.*, 2001). Here we show that ET-1 down-regulates MMP14 and 15 mRNA and protein expression in a dose-dependent manner. This effect is not accounted for by ET-1 affecting trophoblast differentiation, because HLA-G protein, a classical marker of EVT, was unaltered by the treatment (Supplementary data, Fig. S3).

Interestingly, we only detected the active form of MMP14, whereas both the pro- and active forms of MMP15 were observed. Since MT-MMPs are activated by Golgi-associated pro-protein convertases (PCs) (Golubkov *et al.*, 2007), our data suggest that different members of the PC family might be involved in MMP14 and MMP15 activation. We analyzed the ratio between pro- and active-MMP15 as a measure of MMP15 activation. This ratio was lowest in primary trophoblasts from mid first trimester (GW 9 + 10), suggesting that the activation of specific proteases is fine-tuned during the first trimester of pregnancy. However, ET-1 did not alter MMP15 activation in any of these three periods, indicating that ET-1 only regulates MMP15 levels (Supplementary data, Fig. S4).

In first trimester placenta ETRA is located in VT, whereas ETRB is predominantly located in EVT (Cervar-Zivkovic *et al.*, 2011). Blocking ETRB abolished the effect of ET-1 on MMP14 and 15 down-regulation. Contrarily, ETRA blocking augmented this effect, likely due to a higher fraction of ET-1 being bound to ETRB. These results argue for ETRB as the major receptor through which ET-1 induced signals downregulate MMP14 and MMP15.

We used two different assays to address the functional consequences of ET-1 during the first trimester of pregnancy. ET-1 induced a down-regulation of both trophoblast outgrowth from chorionic villi

and trophoblast invasion. The ET-1 concentration (100 nM) used here is similar to other studies assessing biological functions of ET-1 (Chakraborty *et al.*, 2003). Higher ET-1 concentrations had an opposite effect, i.e. stimulated trophoblast invasion (Cervar-Zivkovic *et al.*, 2011), which is in line with the bimodal ET-1 effect observed in a variety of cell systems (Cervar *et al.*, 1996).

We have analyzed the additional effect of TNF-α on ET-1-mediated MMP14 and 15 regulation. TNF-α is an important regulator of trophoblast invasion and is up-regulated in PE (Otun *et al.*, 2011; Mihu *et al.*, 2015). While TNF-α alone did not change MMP14 and MMP15 protein, it enhanced the ET-1 effect on MMP15. Interestingly, TNF-α increases ET-1 expression and *vice versa* (Zhao *et al.*, 2005), suggesting a complex interplay between ET-1 and inflammation, which in turn might fine-tune the trophoblast proteome.

Oxygen regulates MMP expression (Onogi *et al.*, 2011). Placental development during the first trimester occurs in a low oxygen environment (2–3% O_2) (James *et al.*, 2006), and hypoxia (1% O_2) is considered as one of the hallmarks of PE (Ouyang *et al.*, 2009). We observed lower MMP14 and MMP15 protein levels in primary trophoblasts exposed to low oxygen tensions. Interestingly, oxygen also modulated ET-1-mediated MMP14 and 15 down-regulation. Under 2.5% O_2, only MMP15 was down-regulated in the presence of ET-1, pointing that in an *in vivo* oxygen environment, ET-1 might preferentially regulate MMP15 than MMP14. Hypoxia (1% O_2) abolished the effect of ET-1 on both MMPs. We confirmed that an up-regulation of endogenous ET-1 levels in trophoblasts might underlie this observation. The ET-1 promoter is highly responsive to hypoxia (Stow *et al.*, 2011), which might elevate ET-1 levels above its bimodal threshold, and thus act as a negative feedback mechanism.

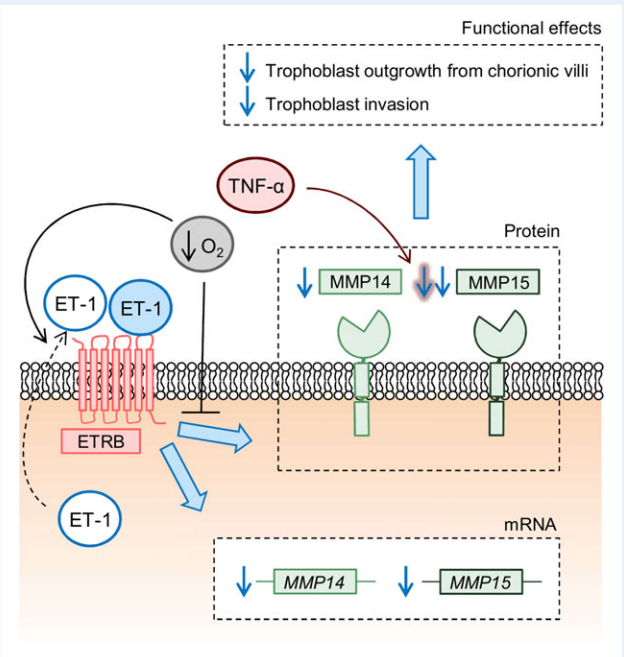

Figure 6 Proposed mechanism of MMP14 and MMP15 regulation by ET-1, TNF-α and O_2 in primary trophoblasts: Upon binding to ETRB exogenous ET-1 (blue filling) down-regulates MMP14 and 15 mRNA expression; This is paralleled by a down-regulation of MMP14 and 15 protein levels; TNF-α enhances ET-1-mediated MMP15 down-regulation, whereas hypoxia abolished the effect of ET-1 on both MMPs by a feedback mechanism up-regulating endogenous ET-1 (white filling).

The major strength of the study is the use of primary human trophoblasts, which differ from first trimester trophoblast cell lines in many properties and also in their response to ET-1 (Bilban *et al.*, 2000). The main limitation of this study is the absence of an in-depth analysis of ET-1 down-stream signaling. The effect of ET-1 on ETR levels was also not characterized. This was not possible because of the limited amount of first trimester placental tissue available for trophoblast isolation. Moreover, the effect of ET-1 on other factors involved in trophoblast invasion cannot be ruled out.

Summary and conclusion

In the present study we have shown that ET-1, one of the molecules up-regulated in PE, alters MMP14 and 15 expression via ETRB in first trimester trophoblasts, and impairs trophoblast functions such as migration and invasion. Our results improve the current knowledge about the molecular mechanisms underlying trophoblast invasion and PE. We have also shown the importance of considering MMP14 and 15 regulation in a more complex environment, where other factors such as inflammation or oxygen might act as modulators of this regulation (Fig. 6). Modulation of the ET/ETR system in animal models of PE is required to confirm our findings *in vivo* and might open new therapeutic strategies for the management of this pathology.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

A.M.M. planned and performed the majority of the experiments and data analysis and wrote the first draft of the manuscript. P.V. performed the explant and transwell invasion assays. J.P. and M.K. planned the explant and transwell invasion assays and critically reviewed the manuscript. H.Y. and G.B. planned the experiments conducted under various oxygen tensions and critically reviewed the manuscript. N.G. and U.L. critically reviewed the manuscript. U.H., G.D. and M.D. contributed to the experimental planning, to data analysis and discussion, and critically reviewed the manuscript.

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Conflict of interest

None declared.

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