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1 **MicroRNA-29 Differentially Mediates Preeclampsia-Dysregulated Cellular**
2 **Responses to Cytokines in Female and Male Fetal Endothelial Cells**

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15 **Short title:** MiR-29 Mediates Endothelial Dysfunction in PE
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25 **Abstract**

26 **Introduction:** Preeclampsia (PE) differentially impairs female and male fetal endothelial cell
27 function which is associated with the increased risks of adult-onset cardiovascular disorders in
28 children born to mothers with PE. However, the underlying mechanisms are poorly defined. We
29 **hypothesize** that dysregulation of microRNA-29a-3p and 29c-3p (miR-29a/c-3p) in PE disturbs
30 gene expression and cellular responses to cytokines in fetal endothelial cells in a fetal sex-
31 dependent manner. **Methods:** RT-qPCR analysis of miR-29a/c-3p was performed on female and
32 male unpassaged (P0) human umbilical vein endothelial cells (HUVECs) from normotensive (NT)
33 and PE pregnancies. Bioinformatic analysis of an RNAseq dataset was performed to identify PE-
34 dysregulated miR-29a/c-3p target genes in female and male P0-HUVECs. Gain- and loss-of-
35 function assays were conducted to determine the effects of miR-29a/c-3p on endothelial
36 monolayer integrity and proliferation in response to TGFβ1 and TNFα in NT and PE HUVECs at
37 passage 1. **Results:** PE downregulated miR-29a/c-3p in male, but not female P0-HUVECs. PE
38 dysregulated significantly more miR-29a/c-3p target genes in female vs. male P0-HUVECs. Many
39 of these PE-differentially dysregulated miR-29a/c-3p target genes are associated with critical
40 cardiovascular diseases and endothelial functions. We further demonstrated that miR-29a/c-3p
41 knockdown specifically recovered the PE-abolished TGFβ1-induced strengthening of endothelial
42 monolayer integrity in female HUVECs, while miR-29a/c-3p overexpression specifically enhanced
43 the TNFα-promoted cell proliferation in male PE HUVECs. **Conclusions:** PE differentially
44 dysregulates miR-29a/c-3p and their target genes associated with cardiovascular diseases- and
45 endothelial function in female and male fetal endothelial cells, possibly contributing to the fetal
46 sex-specific endothelial dysfunction observed in PE.

47

48 **Keywords:** Preeclampsia; MicroRNA-29; Cytokines; Fetal endothelial function; Sexual
49 dimorphisms.

50

51 **Nonstandard abbreviations:** Preeclampsia (PE); Normotensive (NT); Female (F); Male (M);
52 Differentially expressed (DE)

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53 **Background**

54 Preeclampsia (PE) is a hypertensive disorder that complicates 3~8% of all human
55 pregnancies(Anderson *et al.*) and costs billions of dollars to the U.S. healthcare system
56 annually(Stevens *et al.*). PE is a leading cause of fetal and maternal morbidity and mortality during
57 pregnancy(Askie *et al.*; Powe *et al.*, 2011a), and adversely affects a wide range of fetal endothelial
58 function such as cell proliferation, cell migration, monolayer integrity (or permeability), Ca^{++}
59 response, and nitric oxide (NO) production(Wang *et al.*; Powe *et al.*, 2011a; Boeldt *et al.*;
60 Brodowski *et al.*; Zhou *et al.*, 2019; Zhou *et al.*, 2020). To date, the mechanisms underlying such
61 fetal endothelial dysfunction in PE are poorly defined.

62 Pro-inflammatory cytokines are closely involved in the pathogenesis of PE(Raghupathy). For
63 example, tumor necrosis factor-α (TNFα) can elevate blood pressure and induce proteinuria (two
64 hallmarks of PE) in pregnant baboons(Sunderland *et al.*). Elevated maternal circulating TNFα is
65 observed in several forms of pregnancy complications including PE(Wang & Walsh; Benyo *et al.*;
66 Hung *et al.*; Raghupathy). TNFα expression is also significantly increased in human PE
67 placentas(Wang & Walsh), contributing to impaired angiogenic activity in PE(Zhou *et al.*).
68 Similarly, TNFα inhibits cell proliferation(Jiang *et al.*), migration, capillary tube formation(Hsu *et*
69 *al.*), and downregulates endothelial nitric oxide synthase (eNOS)(Kim *et al.*), as well as affects
70 cytokine-induced endothelial leukocyte adhesion molecule expression in human umbilical vein
71 endothelial cells (HUVECs) in vitro(Collins *et al.*; Van Antwerp *et al.*; Mahboubi *et al.*). In addition,
72 transforming growth factor-beta1 (TGFβ1), another growth factor and cytokine also regulates
73 endothelial function, vascular development, and vascular barrier function(ten Dijke & Arthur;
74 Walshe *et al.*, 2009). In PE, TGFβ1 levels are significantly elevated in maternal circulation and
75 are associated with PE-induced endothelial dysfunction(Muy-Rivera *et al.*; Peracoli *et al.*; Lau *et*
76 *al.*, 2013). MicroRNAs (miRNAs) are critical regulators of endothelial function (e.g., proliferation
77 and migration)(Wu *et al.*; Zhou *et al.*, 2017). MiR-29a-3p and miR-29c-3p (refer to as miR-29a/c-
78 3p) are expressed in human endothelial cells and play important roles in maintaining endothelial
79 function(Poliseno *et al.*; Wang *et al.*; Yang *et al.*). Dysregulation of miR-29a/c-3p is associated
80 with cardiovascular diseases (e.g., stroke(Kajantie *et al.*) and heart failure(Thum *et al.*)), which
81 may be partially due to their roles in endothelial cells. Overexpression of miR-29a-3p promotes
82 angiogenesis, whereas knockdown of miR-29a-3p blocks TGFβ1-stimulated angiogenesis in a
83 chick chorioallantoic membrane assay(Wang *et al.*). We have previously reported that PE
84 downregulates miR-29a/c-3p in primary HUVECs and that knockdown of miR-29a/c-3p inhibits
85 growth factor-stimulated fetal endothelial motility in HUVECs(Zhou *et al.*). However, it is unknown

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86 if PE differentially dysregulates the expression and function of miR-29a/c-3p in female (F) and
87 male (M) HUVECs.
88 Increasing evidence has shown that sex is an important regulator of biological processes and
89 cell function. Sexual dimorphisms of fetal endothelial function have been reported in HUVECs
90 from normotensive (NT) pregnancies in cell proliferation, cell viability, tube formation capacity,
91 migration, and endothelial eNOS expression(Addis *et al.*; Lorenz *et al.*). We have also
92 demonstrated that PE differentially dysregulates F and M fetal endothelial transcriptomic profiles
93 and endothelial cell responses (monolayer integrity and proliferation) to growth factors and
94 cytokines (TNFα and TGFβ)(Zhou *et al.*, 2019).
95 In this study, we tested the hypothesis that miR-29a/c-3p is differentially expressed and
96 mediates PE-dysregulated cell responses to cytokines (TNFα and TGFβ) in fetal endothelial cells
97 in a fetal sex-specific manner using HUVECs as a cell model. We determined the PE differentially
98 dysregulated miR-29a/c-3p expression in F and M HUVECs and fetoplacental tissues from NT
99 and PE pregnancies. Bioinformatics and gene ontology analyses were performed to identify PE-
100 dysregulated miR-29a/c-3p target genes/pathways in F and M P0-HUVECs. We also examined
101 the effect of miR-29a/c-3p knockdown and overexpression on endothelial monolayer integrity, cell
102 proliferation, and migration in responses to cytokines in primary F and M HUVECs from NT and
103 PE.
104
105 **Methods**
106 The authors declare that all supporting data are available within the article and its online
107 supplementary files.
108 **Ethical approval**
109 All procedures were conducted in accordance with the Declaration of Helsinki. This research
110 has been approved by the Institutional Review Board of the University of Arizona (Protocol#
111 2104723252) as well as the UnityPoint Health-Meriter Hospital (Madison, WI) and the University
112 of Wisconsin-Madison (Protocol#2004-006). All subjects gave written, informed consent. PE was
113 defined according to standard American College of Obstetricians and Gynecologists
114 criteria(American College of *et al.*, 2013). All PE patients included in this study were late-onset
115 mild PE without major fetal morbidity. Due to the local demographics, all subjects included in this
116 study are Caucasian.
117 **Isolation, purification, and characterization of primary HUVECs**
118 F and M HUVECs were isolated immediately after deliveries of NT and PE pregnancies
119 (Table.1). Cells were then purified using CD31 dynalbeads (Invitrogen, Carlsbad, CA) within ~16h

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120 of culture as previously described(Zhou *et al.*, 2017; Zhou *et al.*, 2019). After purification, 80% of
121 cells (referred to as P0-HUVECs) were immediately snap-frozen in liquid nitrogen until total RNA
122 isolation, and the remaining 20% of cells were cultured to P1 (~5 days of culture). The purity of
123 each cell preparation was evaluated using the DiI-Ac-LDL uptake assay as previously
124 described(Zhou *et al.*, 2017; Zhou *et al.*, 2019). Only cell preparations with ≥ 96% positive DiI-Ac-
125 LDL uptake were used in this study.

126 **Human fetoplacental tissue collection**

127 Human fetoplacental tissues were dissected from the placenta immediately after delivery.
128 After dissection, fetoplacental tissue samples were snap-frozen in liquid nitrogen, stored at -80°C,
129 and then ground to powder in liquid nitrogen followed by RNA isolation.

130 **RNA isolation and quality control**

131 Small RNA-enriched total RNA samples were isolated from P0-HUVECs and placentas using
132 the RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration and quality of each RNA sample
133 were assessed using a NanoDrop™ND-1000 spectrophotometer (NanoDrop Technologies,
134 Wilmington, DE) and Agilent 2100-bioanalyzer (Agilent Technologies, Santa Clara, CA)(Zhou *et*
135 *al.*, 2017; Zhou *et al.*, 2019). Only RNA samples with a high RNA integrity number (>8) were
136 utilized in this study.

137 **RT-qPCR analysis of miR-29a/c-3p in human placentas and P0-HUVECs**

138 To determine the PE-dysregulated miR-29a/c-3p expression in placentas and P0-HUVECs ,
139 RT-qPCR analysis was performed in F and M fetoplacental tissues and P0-HUVECs from NT and
140 PE pregnancies (Table1; n=12-27cell preparations/group/sex) as previously described(Zhou *et*
141 *al.*, 2017). In brief, small RNA fragment enriched total RNA isolated from each sample was reverse
142 transcribed into cDNA using a miScript II RT Kit (Qiagen). RT-qPCR was performed using
143 miScriptSYBR Green PCR Kit (Qiagen) and commercially available miRNA Primer Assays
144 (Table.S1) using a StepOne^{Plus} qPCR system (Life Technologies, Carlsbad, CA). Efficiencies of
145 all target and control miRNA assays were between 90% and 110%. Data were first normalized to
146 an external control (miRTC, Qiagen), followed by normalization to the geometric mean of
147 endogenous control miRNAs (SNORD95, and SNORD96A). The normalized data were then
148 analyzed using the $2^{-\Delta\Delta CT}$ method(Yuan *et al.*, 2006; Zhou *et al.*, 2017).

149 **Bioinformatic analysis of RNAseq dataset on P0-HUVECs**

150 We re-analyzed a previously published RNAseq dataset from P0-HUVECs (NCBI GEO
151 accession: GSE116428(Zhou *et al.*, 2019)) to identify PE dysregulated miR-29a/c-3p target genes
152 in P0-HUVECs (Table.S2&S3). MiR-29a/c-3p target genes were determined using TarBase
153 v.8(Karagkouni *et al.*, 2018) (experiment supported miRNA targets database) and microT-

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154 CDS(Paraskevopoulou *et al.*, 2013) databases. Functional genomics analysis of these PE-
155 dysregulated miR-29a/c-3p target genes was performed to predict the enriched canonical
156 pathways, diseases and biological functions, as well as gene networks using Ingenuity Pathway
157 Analysis (IPA; www.qiagenbioinformatics.com)(Kramer *et al.*, 2014; Zhou *et al.*, 2019).

158 **Knockdown and overexpression of miR-29a/c-3p in F and M P1-HUVECs**

159 Knockdown and overexpression of miR-29a/c-3p were performed using miScript miRNA
160 mimics [Qiagen, refer as miRNA(+)] and miScript miRNA Inhibitor [Qiagen, refer as miRNA(i)]
161 respectively, as described(Ukai *et al.*, 2012; Zhou *et al.*, 2017). Transfection dose and time were
162 pre-determined as described(Zhou *et al.*, 2017) (Fig.S1 and Supplemental Methods). MiRNA
163 mimics and inhibitors were chemically synthesized and modified single-strand RNAs that
164 specifically overexpress and inhibit target miRNA(Ukai *et al.*, 2012; Zhou *et al.*, 2017). In brief,
165 individual primary P1-HUVECs at 50-60% confluence were transfected with miRNA mimic
166 targeting human miR-29a/c-3p [miR-29a/c-3p(+); MSY0000086; Qiagen] and miRNA inhibitor
167 targeting human miR-29a/c-3p [miR-29a/c-3p(i); MIN0000681; Qiagen] using the Qiagen
168 HiPerFect Transfection Reagent for 24hr. Cells transfected with only the transfection reagent and
169 miScript inhibitor negative control were used as the vehicle (Veh) and negative control (NC),
170 respectively(Zhou *et al.*, 2017). RT-qPCR was used to verify the efficiency of miRNA knockdown
171 and overexpression.

172 **Cell functional assays**

173 After successful knockdown and overexpression of miR-29a/c-3p, P1-HUVECs were treated
174 with TNFα (10ng/ml), TGFβ (10ng/ml), or serum-free endothelial culture media (ECMb, control)
175 followed by cell functional assays as previously described(Zhou *et al.*, 2019) (n=5~10 cell
176 preparations/sex/group; Supplemental methods). The dose and duration of TNFα and TGFβ
177 treatments for each cell functional assay were determined based on our previous reports(Zhou *et*
178 *al.*, 2017; Zhou *et al.*, 2019). Endothelial monolayer integrity was determined using the ECIS
179 Zθ+96-well array station (Applied BioPhysics, NY) using 96W10idf plates(Zhou *et al.*, 2019). Cell
180 proliferation was assessed using the CCK-8 kit (Dojindo Molecular Technologies, Rockville,
181 MD)(Zhou *et al.*, 2019).

182 **Statistical analyses**

183 SigmaPlot software (Systat Software., San Jose, CA) was used for statistical analyses. Data
184 are represented as the medians ± standard deviation (SD). Data analyses were performed using
185 the Mann-Whitney Rank Sum Test or Kruskal-Wallis test as appropriate. Differences were
186 considered significant when P <0.05. Benjamini and Hochberg False Discovery Rate (FDR)-

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187 adjustment(Zhou *et al.*, 2017; Zhou *et al.*, 2019) was used for multiple comparison correction as
188 appropriate.
189

190 **Results**

191 **PE differentially dysregulates miR-29a/c-3p expression in F and M placentas and HUVECs.**

192 Compared with NT, PE increased the levels of miR-29a-3p and miR-29c-3p by 72% and
193 106%, respectively, in F, but not M placentas (Fig.1A). Both miR-29a-3p and miR-29c-3p levels
194 were significantly higher in F than in M PE placentas. In contrast, PE decreased the levels of miR-
195 29a-3p and miR-29c-3p by 15% and 24%, respectively, in M but not F P0-HUVECs (Fig.1B).
196 There were no significant differences in miR-29a-3p and miR-29c-3p levels between F and M
197 placentas and P0-HUVECs from NT. However, miR-29a-3p and miR-29c-3p levels were
198 significantly higher in F than in M placentas, while miR-29c-3p levels were higher in F vs. M PE
199 P0-HUVECs.

200 **PE differentially dysregulates miR-29a/c-3p target genes in F and M HUVECs**

201 Re-analysis of our previously published RNAseq dataset on P0-HUVECs (NCBI GEO
202 accession: GSE116428(Zhou *et al.*, 2019)) have identified 2883 (2357 experimentally supported
203 and 526 theoretically predicted, Table.S2) miR-29a/c-3p target genes in P0-HUVECs (Fig. 2B).
204 Among them, 1631 were common target genes of miR-29a/c-3p, while 477 and 775 were miR-
205 29a-3p and miR-29c-3p specific target genes, respectively (Table.S2). Our analysis also showed
206 that PE differentially dysregulated miR-29a/c-3p target genes in F and M P0-HUVECs (Fig.2A-C,
207 Table.S3). A total of 125 miR-29a/c-3p target genes were differentially expressed (DE) between
208 NT-F and NT-M P0-HUVECs (Fig.2B-C, Table.S3), among which two and one genes are located
209 on X- and Y-chromosomes, respectively (Table.S3). In PE-F P0-HUVECs, 110 miR-29a/c-3p
210 target genes were dysregulated with two DE genes located on X-chromosome (Fig.2B-C,
211 Table.S3). In PE-M P0-HUVECs, 28 miR-29a/c-3p target genes were dysregulated with two DE-
212 genes located on the X-chromosome; none of these DE genes located on the Y-chromosome
213 (Fig.2B-C, Table.S3). Only five miR-29a/c-3p target genes (ARC, CILP2, NEFM, GRIN2B, and
214 CHRNA7) were dysregulated in both PE-F and PE-M P0-HUVECs (Fig.2B-C, Table.S3). All these
215 five genes were down-regulated in PE-F, but up-regulated in PE-M P0-HUVECs (Table.S3).

216 **PE differentially dysregulates cardiovascular diseases- and endothelial function-**
217 **associated miR-29a/c-3p target genes in F and M HUVECs**

218 Canonical pathways enrichment analysis on PE-dysregulated genes in F and M P0-HUVECs
219 (Fig.2D and S2) indicated that only one miR-29a/c-3p target canonical pathway (pulmonary
220 fibrosis idiopathic signaling pathway) was significantly dysregulated in both PE-F and PE-M

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221 HUVECs. Nine canonical pathways were enriched only in PE-F HUVECs, and six of these
222 pathways including wound healing signaling pathway and pathogenesis of multiple sclerosis were
223 enriched only in PE-upregulated miR-29a/c-3p target genes in PE-F HUVECs.

224 Diseases and Bio-function enrichment analysis on PE-dysregulated genes in P0-HUVECs
225 (Fig.2E and S3, Table.S4, S5 and S6) further showed that miR-29a/c-3p target genes associated
226 with angiogenesis, atherosclerosis, cell viability, concentration of Ca^{2+}, disorder of blood pressure,
227 obesity, and seizures were dysregulated in PE-F and PE-M HUVECs. Specifically,
228 atherosclerosis, concentration of Ca^{2+}, disorder of blood pressure, and seizures-associated miR-
229 29a/c-3p target genes were only enriched in PE-upregulated genes in M-HUVECs, while
230 concentration of Ca^{2+}, cell viability, and seizures-associated miR-29a/c-3p target genes were only
231 enriched in PE-downregulated genes in F-HUVECs (Fig.S3, Table S5). Additionally, miR-29a/c-
232 3p target genes associated with accumulation of lipid, cellular infiltration, invasion of cells,
233 migration of endothelial cells, chemotaxis, inflammatory response, adhesion of immune cells,
234 recruitment of mononuclear leukocytes, apoptosis of antigen presenting cells, and disruption of
235 microvascular endothelial cells were only enriched in PE-upregulated genes in F-HUVECs.
236 However, miR-29a/c-3p target genes associated with accumulation of extracellular matrix,
237 adhesion of endothelial progenitor cells, concentration of reactive oxygen species, remodeling of
238 blood vessel, vasodilation of pulmonary artery, hypertension, and stroke were only enriched in
239 PE-upregulated genes in M-HUVECs.

240 Upstream regulator analysis on PE-dysregulated genes in P0-HUVECs (Table.2, Table.S7)
241 revealed that TNF-, TGFB1-, IFNG-, IL1B-, MYC-, AGT-, FOXO1-, MAPK1-, PDGF BB-, F2-, and
242 CSF2-regulated miR-29a/c-3p target genes were enriched in both PE-F and PE-M HUVECs, in
243 which PE-F HUVECs has more PE-dysregulated miR-29a/c-3p target genes in all these gene
244 networks than PE-M cells. In addition, NFκB-, IL33-, and IL1-regulated miR-29a/c-3p target genes
245 were uniquely enriched in PE-F HUVECs.

246 **MiR-29a/c-3p differentially regulate endothelial monolayer integrity in F and M HUVECs**
247 **from NT and PE**

248 Compared with NC, miR-29a/c-3p(+) at 10nM significantly increased (> 340%) the level of
249 both miR-29a-3p and miR-29c-3p in HUVECs 24h after transfection and this overexpression
250 maintained for up to 72h (Fig.S1). We previously reported that miR-29a/c-3p(i) at 50nM
251 significantly decreased the levels of both miR-29a-3p and miR-29c-3p in HUVECs after 24-72h
252 of transfection(Zhou *et al.*, 2017). Hence miR-29a/c-3p(+) at 10nM and miR-29a/c-3p(i) at 50nM
253 were utilized in all miR-29a/c-3p overexpression and knockdown experiments in this study.

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254 Veh and NC did not alter the endothelial monolayer integrity in all cell groups treated with
255 ECMb, TGFβ1, and TNFα (Fig.3 and Fig.S4).
256 Compared with Veh and NC, miR-29a/c-3p(+) time-dependently increased electrical
257 resistance (strengthening endothelial monolayer integrity) in NT-F HUVECs, but not in NT-M PE-
258 F and PE-M HUVECs (Fig.3A). Specifically, in NT-F HUVECs, miR-29a/c-3p(+) strengthened
259 endothelial monolayer integrity, beginning at 7h and reached a maximum increase of ~15% at
260 24h. This miR-29a/c-3p(+)-enhanced monolayer integrity was lost in PE-F HUVECs.
261 Compared with Veh and NC, miR-29a/c-3p(i) time-dependently decreased electrical
262 resistance (weakening endothelial monolayer integrity) only in NT-M, but not in NT-F, PE-F, and
263 PE-M HUVECs in ECMb (Fig.3A). Specifically, in NT-M HUVECs, miR-29a/c-3p(i) weakened
264 endothelial monolayer integrity, beginning at 10h, reached a maximum of ~9% decrease at 16h
265 and maintained this level through 25h.

266 **MiR-29a/c-3p differentially regulate endothelial monolayer integrity in response to TGFβ1**
267 **in F and M HUVECs from NT and PE**

268 Compared with ECMb, TGFβ1 time-dependently strengthened endothelial monolayer
269 integrity in NT-F HUVECs, but not in NT-M, PE-F, and PE-M HUVECs. Specifically, TGFβ1
270 strengthened endothelial monolayer integrity in NT-F transfected with Veh and NC, starting at 15h
271 and reaching 14% and 9% enhancement at 25h, respectively (Fig.3B). MiR-29a/c-3p(+) further
272 enhanced the TGFβ1-strengthened endothelial monolayer integrity in NT-F HUVECs, starting at
273 16h and reaching 21% enhancement at 25h. The TGFβ1-strengthened endothelial monolayer
274 integrity in F HUVECs was abolished in PE-F cells, while miR-29a/c-3p(i) recovered this TGFβ1-
275 strengthened endothelial monolayer integrity in PE-F HUVECs (reaching 15% enhancement at
276 25h). MiR-29a/c-3p(i) did not alter endothelial monolayer integrity in NT-F, NT-M, and PE-M
277 HUVECs.
278 Compared to ECMb, TNFα significantly decreased the endothelial monolayer integrity in NT-
279 F, NT-M, PE-F, and PE-M HUVECs (Fig.S4). MiR-29a/c-3p(i) and miR-29a/c-3p(+) did not alter
280 the TNFα- weakened endothelial monolayer integrity in NT-F, NT-M, PE-F, and PE-M HUVECs.

281 **MiR-29a/c-3p differentially regulate TNFα-induced cell proliferation in F and M HUVECs**
282 **from PE**

283 Veh and NC did not alter the cell proliferation in all cell groups treated with ECMb, TGFβ1,
284 and TNFα. Compared with Veh and NC control, miR-29a/c-3p(+) and miR-29a/c-3p(i) did not alter
285 the cell proliferation in response to ECMb and TGFβ1 in NT-F, NT-M, PE-F, and PE-M (Fig.4).
286 Compared to ECMb control, TNFα promoted cell proliferation in PE-F (159%) and NT-M
287 (153%) HUVECs (Fig.4C). Compared to Veh and NC, miR-29a/c-3p(+) further promoted the cell

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288 proliferation in PE-M HUVECs (181% of ECMb control), while MiR-29a/c-3p(i) did not affect the
289 endothelial proliferation responses to TNFα in all HUVECs groups.
290

291 **Discussion**

292 In this study, we have demonstrated for the first time that PE dysregulates miR-29a/c-3p in
293 fetoplacental tissues and primary human fetal endothelial cells (HUVECs) in a fetal sex-specific
294 manner. We have further shown fetal sex-specific dysregulation of cardiovascular diseases- and
295 endothelial function-associated miR-29a/c-3p target genes in F and M fetal endothelial cells from
296 PE. Moreover, miR-29a/c-3p overexpression and knockdown differentially affect PE-dysregulated
297 fetal endothelial cell responses to cytokines in F and M HUVECs. These data indicate fetal sex-
298 specific roles of miR-29a/c-3p in PE-dysregulated transcriptomic profiles and cell functions in fetal
299 endothelial cells.

300 Expression of miR-29a/c-3p in human placentas are significantly higher in the third trimester
301 than that in the first trimester(Gu *et al.*, 2013), suggesting miR-29a/c-3p play important roles in
302 placental development and function. Our current finding that PE upregulated miR-29a/c-3p levels
303 in F, but not M fetoplacentas agrees with a previous report that miR-29a-3p is elevated in maternal
304 plasma from mild PE patients(Li *et al.*, 2013), indicating fetal sex-specific expression patterns of
305 miR-29a/c-3p in PE placentas. To date, although we do not know the exact cell contribution to
306 this PE-dysregulated miR-29a/c-3p expression, this fetal sex-specific upregulation of miR-29a/c-
307 3p in PE placentas suggests that this dysregulation may contribute to the PE-impaired placental
308 functions.

309 The current observation that PE only downregulates miR-29a/c-3p expression in M, but not
310 in F HUVECs extends our previous report(Zhou *et al.*, 2017; Zhou *et al.*, 2019) and supports our
311 hypothesis that PE differentially regulates miR-29a/c-3p expression in F and M HUVECs.
312 However, this differential expression pattern in HUVECs is opposite to that in placentas, indicating
313 different regulation of miR-29a/c-3p in placental tissues and HUVECs. We have previously
314 reported that F HUVECs are transcriptionally more responsive to PE than their M
315 counterparts(Zhou *et al.*, 2019). In this study, we found that PE-dysregulated ~4.5-fold of miR-
316 29a/c-3p target genes in F HUVECs (125 genes) than in M HUVECs (28 genes). These data
317 imply that while miR-29a/c-3p has a critical role in PE-dysregulated transcriptomes in F and M
318 HUVECs, F HUVECs are more susceptible to miR-29a/c-3p regulation in PE. It is noteworthy that
319 there are only five common PE-dysregulated miR-29a/c-3p target genes observed in F and M
320 cells. However, these five DE genes had opposite regulation directions in F and M HUVECs: all
321 were down-regulated in F but up-regulated in M HUVECs from PE. These DE genes include

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322 Activity Regulated Cytoskeleton Associated Protein (ARC) and Cartilage Intermediate Layer
323 Protein 2 (CLIP2), both of which are important for intercellular microRNA transportation via
324 extracellular exosome(Pastuzyn *et al.*, 2018; Hu *et al.*, 2020). Thus, fetal sex-specific
325 dysregulation of these intercellular microRNA transportation-related genes may contribute to PE-
326 dysregulated miR-29a/c-3p in HUVECs and fetoplacental tissues. This notion is consistent with
327 our previous report that PE, in general, dysregulates more cardiovascular/endothelial function-
328 associated pathways and biological functions in F than in M HUVECs(Zhou *et al.*, 2019), as well
329 as our observation in the current study that miR-29c-3p expression in PE-F is higher than in PE-
330 M HUVECs.

331 Many of the PE-dysregulated miR-29a/c-3p target pathways and biological functions (e.g.,
332 cell movement, cell viability, angiogenesis, and concentration of Ca^{2+}) in HUVECs are associated
333 with PE-induced fetal endothelial dysfunction (e.g., reduced cell migration, and impaired calcium
334 signaling) and vascular-related disorders (e.g., atherosclerosis, disorder of blood pressure,
335 obesity, and seizures)(Wang *et al.*; Powe *et al.*, 2011a; Boeldt *et al.*; Brodowski *et al.*; Zhou *et al.*,
336 2019; Zhou *et al.*, 2020). These data suggest that miR-29a/c-3p play important roles in vascular
337 functions during pregnancy and PE-offspring, as PE-offspring are known to face increased risks
338 of cardiovascular and metabolic disorders(Kajantie *et al.*, 2009; Ryckman *et al.*, 2013).
339 Furthermore, PE uniquely dysregulated many endothelial function-associated miR-29a/c-3p
340 target pathways and biological processes in F and M HUVECs. Notably, wound healing signaling,
341 inflammatory response, and obesity were only enriched in PE-upregulated miR-29a/c-3p target
342 genes in F HUVECs, while hypertension, stroke, and adhesion of endothelial progenitor cells were
343 enriched only in PE-upregulated miR-29a/c-3p target genes in M HUVECs (Fig. 2D&E,
344 Fig.S2&S3). In agreement with a previous report that dysregulated miRNA profiles in HUVECs
345 are associated with dermal microvascular density neonates(Yu *et al.*, 2018), These observations
346 suggest that PE-dysregulated miR-29a/c-3p involve in the *in-utero* programming of fetal
347 endothelial cells and prime PE offspring for higher risks of cardiovascular diseases later in their
348 life.

349 The current finding that PE dysregulates much more inflammatory- and immune responses
350 -associated genes (e.g., inflammatory response, adhesion of immune cells, apoptosis of antigen-
351 presenting cells, NFkB, IL33, and IL1) in F than M HUVECs suggest a fetal sex-specific role of
352 miR-29a/c-3p in the inflammatory response in PE. This is consistent with reports showing that the
353 miR-29 family participates in the immunological responses after virus infections (HIV-1 and
354 SARS-CoV-2)(Abel *et al.*, 2021; Saulle *et al.*, 2021).

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355 Consistent with our bioinformatics analysis showing that PE-dysregulated miR-29a/c-3p
356 target genes/pathways are highly associated with inflammatory responses in F and M HUVECs
357 (Fig. 2), we observed that PE differentially regulated cell responses to inflammatory-related
358 cytokines (TGFβ1 and TNFα) in F and M HUVECs. For instance, in agreement with our previous
359 report(Zhou *et al.*, 2019), TGFβ1 strengthens endothelial monolayer integrity only in NT F-
360 HUVECs, which is abolished in PE, while TNFα decreases endothelial monolayer integrity in
361 HUVECs. These data confirm the vital role of TGFβ1 and TNFα in fetal endothelial functions.

362 We observed that miR-29a/c-3p overexpression increased basal endothelial monolayer
363 integrity only in NT-F, and miR-29a/c-3p knockdown decreased basal endothelial monolayer
364 integrity only in NT-M cells, whereas this fetal sex-specific differential regulation was lost in PE-F
365 and PE-M HUVECs. We also showed that miR-29a/c-3p overexpression further enhanced
366 TGFβ1-strengthened endothelial monolayer integrity in F but not in M NT-HUVECs, while this
367 fetal sex-specific regulation disappeared in PE HUVECs. Furthermore, miR-29a/c-3p knockdown
368 recovered the TGFβ1-enhanced endothelial monolayer integrity in PE-F HUVECs but did not
369 affect the monolayer integrity in PE-M cells. Together with our bioinformatic data that PE
370 dysregulated more TGFβ1-regulated miR-29a/c-3p target genes in PE-F than PE-M HUVECs,
371 these differential regulations implicate the fetal sex-specific importance of miR-29a/c-3p in
372 maintaining basal and TGFβ1-induced endothelial monolayer integrity responses in HUVECs.
373 Overall, PE-F HUVECs are more susceptible to miR-29a/c-3p regulated endothelial monolayer
374 integrity responses than PE-M cells.

375 Overexpression and knockdown of miR-29a/c-3p do not alter the basal cell proliferation as
376 well as cell proliferation in response to TGFβ1 and TNFα in NT HUVECs, indicating that miR-
377 29a/c-3p is not critical to maintain the basal as well as TGFβ1- and TNFα-regulated cell
378 proliferation responses in NT HUVECs. However, overexpression of miR-29a/c-3p recovers the
379 TNFα-induced cell proliferation in PE-M and brings it to similar levels of NT-M cells. It appears
380 that PE-M HUVECs are more responsive to the miR-29a/c-3p regulated cell proliferative
381 responses than PE-F cells.

382 As the human umbilical vein carries oxygenated blood from the placenta to the growing fetus
383 during pregnancy, HUVECs are a unique cell population that is directly exposed to altered
384 humoral factors derived from placenta and maternal circulation and share many features of artery
385 endothelial cells(Inoue *et al.*, 1998; Lang *et al.*, 2008; Jiang *et al.*, 2013). Although a direct
386 relationship between endothelial dysfunction observed in primary HUVECs and specific long-term
387 cardiovascular risks in the offspring remain elusive, the emerging evidence has shown that
388 dysregulated miRNA expression and dysfunction of HUVECs from hypertensive pregnancies

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389 including PE are associated with vascular dysfunction in offspring(Powe *et al.*, 2011b; Staley *et*
390 *al.*, 2015; Yu *et al.*, 2018).
391

392 **Conclusions**

393 Our data have demonstrated that PE differentially dysregulates the expression of miR-29a/c-
394 3p, miR-29a/c-3p target genes/pathways, and miR-29a/c-3p-associated endothelial cell function
395 in response to TGFβ1 and TNFα in F and M HUVECs. These fetal sex-specific dysregulations
396 may contribute to fetal sex-specific vascular dysfunction in PE and PE-associated adult-onset
397 cardiovascular diseases in PE offspring.

398 **Perspectives**

399 To date, there are limited therapeutic options for PE-induced fetal endothelial dysfunction
400 due to our poor understanding of cellular and molecular mechanisms underlying PE. Here we
401 reported fetal sexual dimorphic regulation of miR-29a/c-3p and miR-29a/c-3p target
402 genes/pathways in HUVECs in PE. We also demonstrated fetal sex-specific dysregulation of miR-
403 29a/c-3p-associated cellular responses to TGFβ1 and TNFα in PE HUVECs. These sexual
404 dimorphisms of PE-dysregulated miR-29a/c-3p and their target genes/pathways may allow the
405 discovery of novel fetal sex-specific therapeutic targets and risk predictors for adult-onset
406 cardiovascular diseases in children born to PE mothers.
407

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421 **Conflict of Interest/Disclosure Statement**

422 The authors have no conflict of interest.

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Table.1. Patient Demographics*

Diagnosis	Fetal sex	N	Maternal BMI	Maternal age (years)	Gestational age (weeks)	Systolic BP (mmHg)	Diastolic BP (mmHg)	Protein/ Creatinine ratio (mg/ml)	Fetal weight (gram)	Apgar Score (5min)
NT	F	27	24.7 ± 0.6	32 ± 0.9	39.6 ± 0.2	109 ± 2.0	70 ± 1.8	N/A	3459 ± 73.6	9 ± 0.1
PE	F	15	24.9 ± 0.6	33 ± 1.5	37.3 ± 0.4	148 ± 4.3	90 ± 2.1	0.5 ± 0.5	3030 ± 143.4	9 ± 0.3
			P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05		P < 0.05	P > 0.05
NT	M	20	24.1 ± 0.6	30.5 ± 1	39.3 ± 0.3	118 ± 3.3	73 ± 3.5	N/A	3645 ± 86.5	9 ± 0.1
PE	M	13	24.7 ± 0.7	30 ± 1.2	37.6 ± 0.5	143 ± 3.2	91 ± 2.7	0.6 ± 0.4	3150 ± 115.2	9 ± 0.2
			P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05		P < 0.05	P > 0.05

592 *BP: Blood Pressure; NT: Normotensive pregnancy; PE: Preeclampsia. All subjects are Caucasian.

593

Table.2. PE-dysregulated miR-29a/c-3p target gene network in female and male P0-HUVECs*

Gene Network	PE vs. NT Female P0-HUVECs		PE vs. NT Male P0-HUVECs	
	P-value	# Of miR-29a/c-3p Target in DE-Genes	P-value	# Of miR-29a/c-3p Target in DE-Genes
TNF-Regulated Genes	1.35E-14	38	3.36E-04	9
TGFB1-Regulated Genes	1.81E-13	36	7.83E-07	12
IFNG-Regulated Genes	2.39E-10	28	1.65E-03	7
IL1B-Regulated Genes	4.06E-08	21	4.10E-02	4
NFkB-Regulated Genes	1.06E-11	20	-	-
MYC-Regulated Genes	2.63E-04	16	3.25E-03	6
AGT-Regulated Genes	2.71E-05	15	6.55E-04	6
IL33-Regulated Genes	5.62E-07	12	-	-
FOXO1-Regulated Genes	1.36E-05	11	2.24E-03	4
MAPK1-Regulated Genes	1.60E-06	11	9.93E-03	3
PDGF BB-Regulated Genes	6.67E-07	11	7.78E-03	3
F2-Regulated Genes	8.76E-07	10	4.43E-02	2
IL1-Regulated Genes	6.07E-06	10	-	-
CSF2-Regulated Genes	1.86E-04	10	2.42E-02	3

594 *DE: Differentially expressed. NT: Normotensive pregnancy; PE: Preeclampsia; P0: Passage 0.

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596 **Figures and Legends**

597

598 **Figure 1. PE differentially dysregulates miR-29a/c-3p in F and M placentas (A) and P0-**
599 **HUVECs (B).** (A) Human placentas were collected immediately after delivery (NT-F, n=18; PE-F,
600 n=12; NT-M, n=20; PE-M, n=14). (B) Individual primary P0-HUVECs preparations were isolated
601 immediately after delivery and isolated within 16h (NT-F, n=27; PE-F, n=15; NT-M, n=20; PE-M,
602 n=13). Data are expressed as fold of the corresponding NT-F group ($P < 0.05$, Mann-Whitney
603 Rank Sum Test). *Differ between PE vs. NT in same fetal sex; [Δ]Differ between F and M within the
604 same diagnostic group (PE or NT).

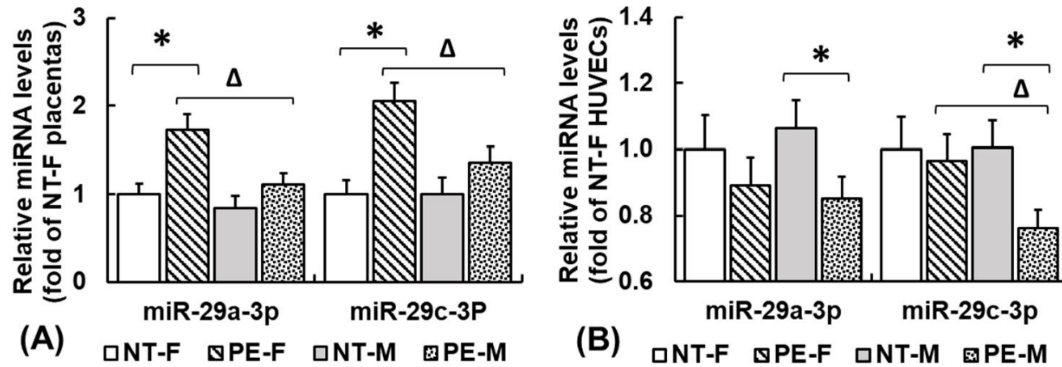

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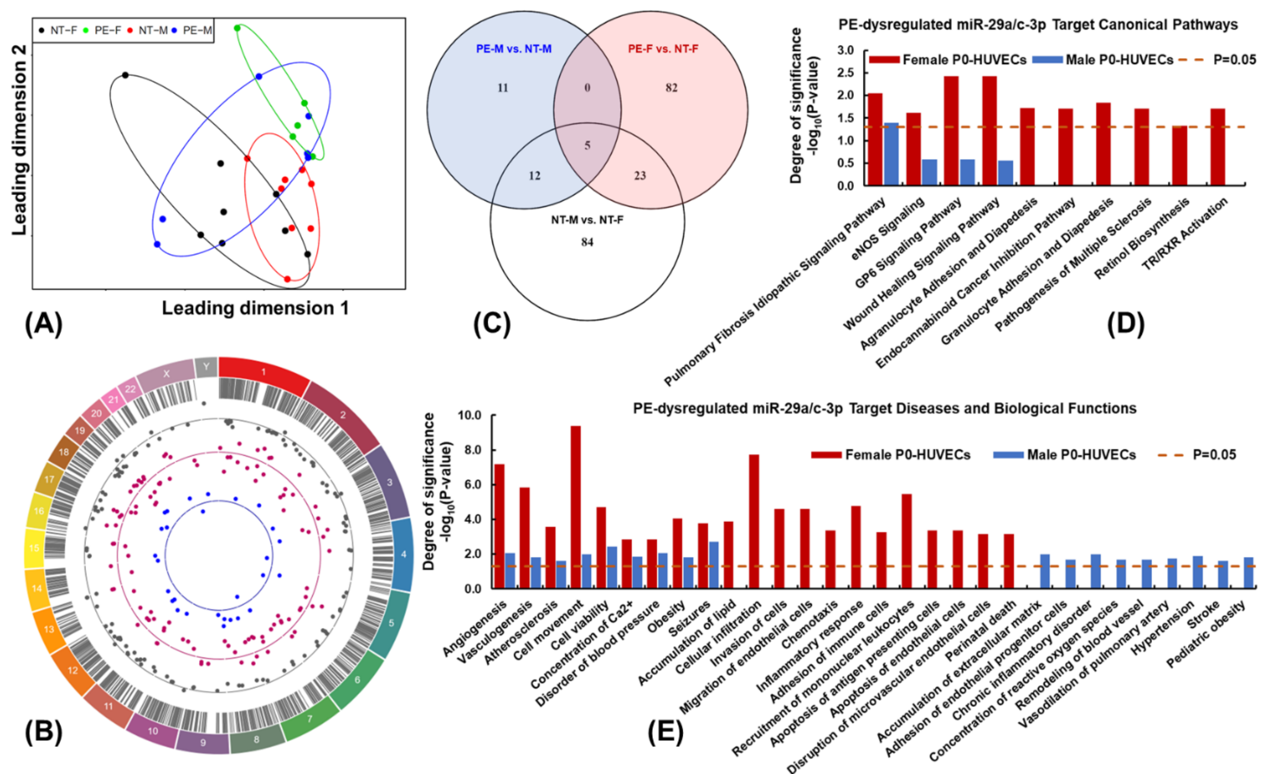
Figure 2. PE differentially dysregulates miR-29a/c-3p target genes. (A) Multi-Dimensional Scaling (MDS) plot representing the similarity and disparity among samples based on expression patterns of miR-29a/c-3p target genes. Each dot represents one biological sample. Distance between dots representing the differences of miR-29a/c-3p target genes expression profiles among samples. Eclipse shows the clustering of samples. **(B)** Circos plot illustrating the chromosomal position of DE miR-29a/c-3p target genes between NT-M vs. NT-F (grey dots, 125 DE-genes), PE-F vs. NT-F (pink dots, 110 DE-genes), and PE-M vs. NT-M (blue dots, 28 DE-genes). Each dot represents one gene. The numbers and letters in the outer ring indicate the chromosomal location. For each scatter plot track, dots outside and inside of the centerline are up- and down-regulated miR-29a/c-3p target genes by PE, respectively. **(C)** Overlap of DE miR-29a/c-3p target genes in NT-M vs NT-F, PE-F vs. NT-F, and PE-M vs. NT-M. Preeclampsia differentially dysregulated **(D)** canonical pathways-, as well as **(E)** diseases and biological functions-associated miR-29a/c-3p target genes in F and M P0-HUVECs. Significant enrichments were determined using IPA software ($P < 0.05$, Fisher's exact test followed with BH-FDR multiple test correction).



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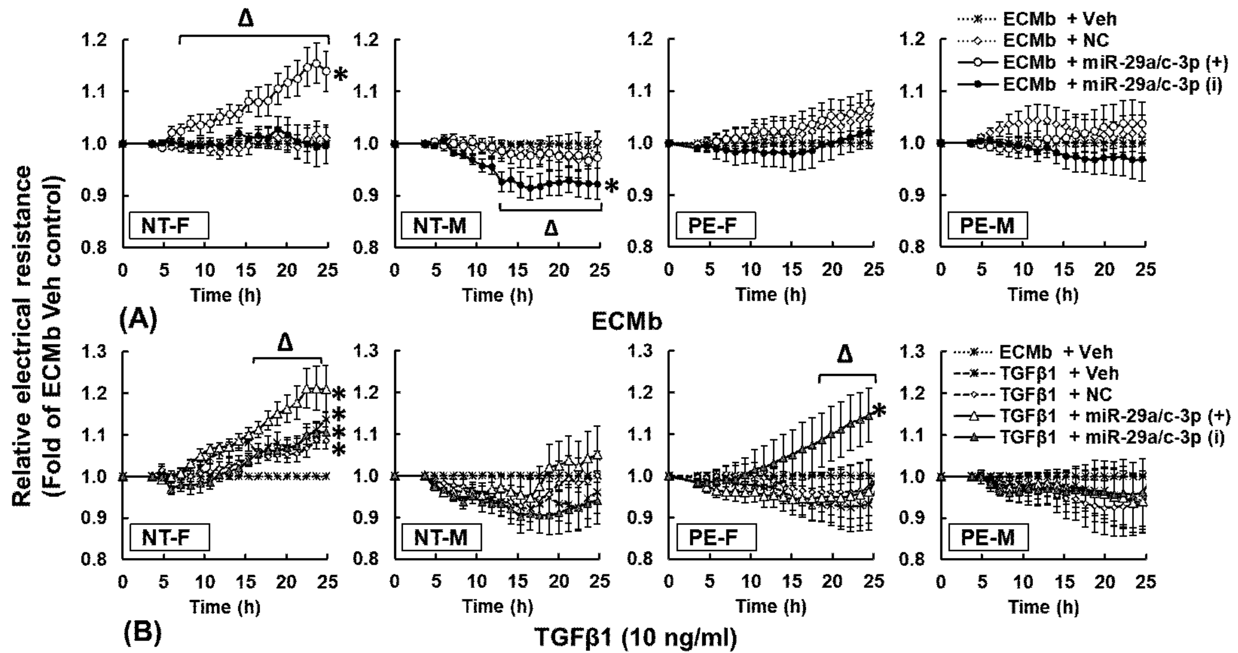
622 **Figure 3. MiR-29a/c-3p differentially regulate basal endothelial monolayer integrity in F and**
623 **M HUVECs from NT and PE.** Cells were transfected with miR-29a/c-3p(+), miR-29a/c-3p(i),
624 Negative Control (NC), or Vehicle (Veh), and then cultured until confluence (24-28h). After 6-8 hr
625 of serum starvation, confluent cells were treated with ECMb (serum-free control), TGFβ1 (10
626 ng/ml), or TNFα (10 ng/ml) for 25h. Electrical resistance at 4000Hz was constantly recorded. Data
627 are expressed as medians ± SEM fold of corresponding Vehicle control at the corresponding time.
628 *Differ (*P*<0.05) from Veh control in ECMb; ΔDiffer from corresponding Veh and NC control
629 groups. (*P*<0.05, Kruskal-Wallis test; n = 5-10 cell preparations/sex/group)



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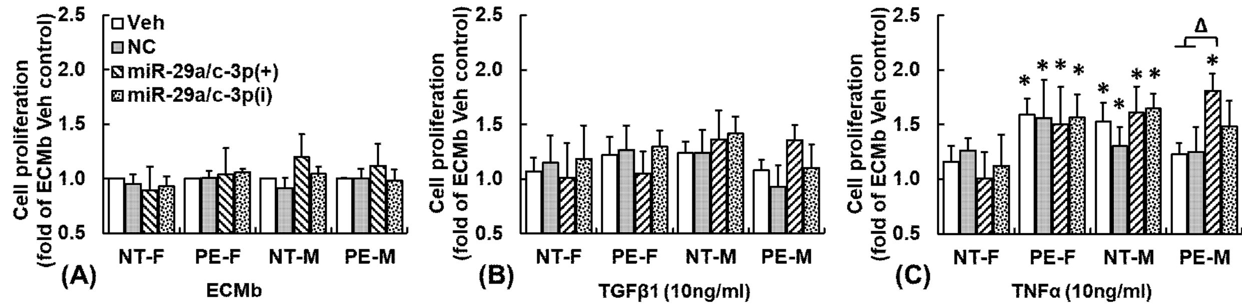
631 **Figure 4. Effects of miR-29a/c-3p on cell proliferation in F and M P1-HUVECs from NT and**
632 **PE.** Sub-confluence cells were transfected with Veh, miR-29a/c-3p(+), or miR-29a/c-3p(i) for 24h.
633 After 8 hr of serum starvation, sub-confluent cells were treated with ECMb [serum free control;
634 **(A)**], TGFβ1 [10 ng/ml; **(B)**], or TNFα [10ng/ml; (**C)**] for 48h. Data are expressed as medians ±
635 SEM fold of ECMb Vehicle control in NT-F. *Differ from corresponding Veh and NC controls in
636 ECMb; ΔDiffer from corresponding NC and Veh control groups within the same treatment.
637 (P<0.05, Kruskal-Wallis test; n=5-8 cell preparations/sex/group).



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