Accepted Manuscript

Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression *in vitro*

J.I. Kalisch-Smith, J.E. Outhwaite, D.G. Simmons, M. Pantaleon, K.M. Moritz

PII: S0143-4004(16)30486-6

DOI: 10.1016/j.placenta.2016.08.080

Reference: YPLAC 3449

To appear in: *Placenta*

Received Date: 17 May 2016

Revised Date: 29 July 2016

Accepted Date: 19 August 2016

Please cite this article as: Kalisch-Smith JI, Outhwaite JE, Simmons DG, Pantaleon M, Moritz KM, Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression *in vitro*, *Placenta* (2016), doi: 10.1016/j.placenta.2016.08.080.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

provided by University of Queensland eSpace
View metadata, citation and similar papers at core.ac.uk
brought to you by CORE

ACCEPTED MANUSCRIPT

1 *Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression in*
2 *vitro.*

3

4 Kalisch-Smith, J.I, Outhwaite, J.E., Simmons, D.G., Pantaleon, M., Moritz, K.M.

5

6 School of Biomedical Sciences, University of Queensland, QLD 4072, Australia

7

8 **Corresponding author**

9 Karen Moritz

10 k.moritz1@uq.edu.au
11 School of Biomedical Sciences
12 The University of Queensland,
13 St Lucia, Qld, 4072
14 Australia
15
16

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

17 **Placenta short communication**

18

19 **Abstract**

20 Maternal alcohol consumption is common prior to pregnancy recognition and in the rat
21 results in altered placental development and fetal growth restriction. To assess the effect of
22 ethanol (EtOH) exposure on the differentiation of trophoblast stem (TS) cells, mouse TS lines
23 were differentiated *in vitro* for 6 days in 0%, 0.2% or 1% EtOH. This reduced both
24 trophoblast survival and expression of labyrinth and junctional zone trophoblast subtype-
25 specific genes. This suggests that fetal growth restriction and altered placental development
26 associated with maternal alcohol consumption in the periconceptional period could be
27 mediated in part by direct effects on trophoblast development.

28

29

30 **3-5 Key Words:** Ethanol, Placenta, Differentiation, Programming, Apoptosis

31

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

32 **1. Introduction**

33 Maternal perturbations during early pregnancy, including a low protein diet [1-3],
34 undernutrition [4,5], or alcohol (EtOH) exposure [6,7], can result in fetal growth restriction
35 and programming of adult disease. Alcohol is a common exposure during pregnancy, with
36 current statistics being 47-58% of all pregnancies [11,12]. Consumption of 5+ standard
37 drinks has also been reported in the period prior to pregnancy recognition (14%) [11,12]. *In*
38 *vivo* rodent models of EtOH exposure during the periconception period [6] and intermittently
39 throughout gestation [13,14], have shown alterations to placental structure and expression of
40 metabolic transporters. This is of interest as normal formation and function of the placenta is
41 a critical determinant of fetal growth. Derived from the trophectoderm of the pre-
42 implantation embryo, trophoblast cells contribute the majority of cells within the mature
43 placenta and consist of a number of unique cell types with diverse morphologies and
44 functions [8]. The definitive chorioallantoic placenta is organised into two zones containing
45 specialised trophoblast cell types; the junctional zone which has a structural and endocrine
46 role, while the labyrinth zone contains the fetal and maternal vasculature and is the location
47 of nutrient exchange [10]. Perturbations during pregnancy often result in modifications to
48 placental growth [2,5] and zonal allocation [6] in late gestation, however few studies have
49 determined whether alterations to trophoblast differentiation in early pregnancy may be
50 mediating these effects. We have previously reported that periconceptional EtOH exposure
51 prior to implantation in the rat causes an increase in glycogen trophoblasts (GlyT) in the
52 junctional zone during late gestation [6]. In addition, high dose (18-37% vol/vol) EtOH
53 exposure from implantation until close to term (E6-E18) reduced invasion of trophoblast cells
54 into the maternal decidua and caused labyrinth disorganisation [14]. However, it is unknown
55 if placental defects are due to direct exposure of the trophoblast stem cells of the
56 trophectoderm to EtOH within the uterine cavity, or via other indirect mechanisms such as

ACCEPTED MANUSCRIPT

57 interactions with altered uterine cells. Here we utilise an *in vitro* model of differentiating
58 mouse trophoblast stem (TS) cells to examine the direct effects of EtOH on proliferation and
59 differentiation.

60

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

61 **2. Methods**

62 *2.1 TS cell culture*

63 Murine TS cells (EGFP line) were maintained as previously described [15]. Cells were
64 seeded at $5x10^4$, and differentiated in 0% (control), 0.2%, or 1% EtOH in TS media. To
65 assess cell proliferation, cells were lifted off the culture plates and counted using a
66 hemocytometer on days 2, 4 and 6 of culture (*N*=3/treatment). Gene expression analysed on
67 day 6 (*N*=9/treatment, 3 technical replicates per set). Media was changed every 2 days
68 including the addition of fresh EtOH. The 0.2% EtOH dose was based on the maximum
69 blood alcohol concentration from 12.5% v/v EtOH exposure in Sprague Dawley dams [6]
70 whilst the 1% EtOH dose has been used previously in culture [24]. In culture, as much as
71 50% evaporation occurs by 8 hours and 80-90% evaporation by 24h in tissue culture plates
72 [18].

73 *2.2 RNA extraction and qRT-PCR*

74 RNA isolation and qRT-PCR were carried out as previously described [24]. Markers specific
75 to labyrinth or junctional zone trophoblasts were analysed for gene expression relative to
76 *Rn18s* using the ΔΔCt method [17] (primer sequences in *Supplemental Table 1).*

77 *2.3 Histological assessment of trophoblast number*

78 Fluorescent *in situ* hybridisation (FISH) localised *Tpbpa* and *Prl7a2* positive trophoblasts
79 (*N*=3-4/treatment) from day 6 cultures [8], see supplemental file for more detail. Fluorescein
80 or DIG-labelled probes were made following manufacturer's instructions (Roche) with
81 primer sequences as described previously [8,20]. Prior to use on cultured cells, RNA probes
82 were tested on either E12.5 or E14.5 mouse placenta fixed in 4% paraformaldehyde overnight
83 to confirm subtype-specific expression. Coverslips were imaged on an inverted microscope

ACCEPTED MANUSCRIPT

84 (Leica) in 20x20 frames, 20x magnification, with all cells being utilised within this area for
85 calculation. Cells were counted using ImageJ (NIH).

86 *2.4 Statistical analyses*

87 Mean relative gene expression of treatments were standardised to the control group for each
88 line. Expression data, proliferation and FISH counts were analysed by one-way ANOVAs to
89 compare treatments. Tukey's post-hoc tests were used where appropriate, and when data was
90 not normally distributed, a non-parametric Kruskal-Wallis test was used. Statistical
91 differences: *$P<0.05$, **$P<0.01$.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

92 **3. Results and Discussion**

93 To determine the mechanism by which EtOH exposure during early pregnancy may be
94 mediating altered placental growth and function, we explored the direct impact of EtOH on
95 TS cell proliferation and differentiation. To investigate EtOH exposure at a physiological
96 level, 0.2% EtOH was chosen as this was the peak blood alcohol content found in a model of
97 *in vivo* alcohol exposure in the rat at 30 minutes after initial consumption [6]. In addition,
98 0.2% EtOH is also the maximum dose that the TS cells would be exposed to when the media
99 is applied, and due to alcohol evaporation [18], would not be sustained at this level over the
100 entire 6 days of treatment. In addition, TS cells express a well-validated set of genetic
101 markers enabling identification of individual placental trophoblast cell types in the mouse [8].
102 This presents an opportunity to relate alterations in gene expression to potential changes in
103 differentiation.

104 **3.1 Cell counts**

105 Proliferation rates were first characterised to determine the effects of EtOH over the course of
106 TS cell differentiation. While no differences in cell count were found between treatments on
107 day 2 or 4, marked reductions in cell counts in both the 0.2% (48% reduction) and 1% EtOH
108 groups (44% reduction) were observed on day 6 (Fig.1A). This time point coincides with
109 terminal differentiation of trophoblasts, as expression of stem cell markers are rapidly lost by
110 day 2 of culture and expression of markers of mature cell types is just commencing on day 4
111 [19]. This result is consistent with studies that demonstrate reduced proliferation in response
112 to 20-40mM (0.09-0.18%) EtOH in cultured human cytotrophoblasts (first trimester villous
113 explants and BeWo cells) [16]. However, our observation of decreased cell numbers at day 6
114 of differentiation is unlikely the result of decreased proliferation, but more likely represents a
115 loss of cell viability. This is supported by the observation of significant floating cells in the

ACCEPTED MANUSCRIPT

116 treatment but not control media on day 6 (data not shown). In addition, human
117 cytotrophoblasts undergo apoptosis when exposed to EtOH [21,22]. To determine whether
118 this also occurs in mouse trophoblasts exposed to EtOH, we analysed the expression profiles
119 of pro- and anti-apoptotic markers (*Bax* and *Bcl2* respectively) from days 2-6 of culture. No
120 changes were found to either gene on days 2 or 4 (data not shown). However, on day 6, whist
121 no change was found to *Bax* expression, *Bcl2* expression was reduced by EtOH (see
122 *Supp.Fig.1A,B*), suggesting a perturbed balance between pro- and anti-apoptotic signals. This
123 may extend to an increase in apoptosis, however, to determine this definitively would require
124 histological analysis. Collectively, these data indicate that placental trophoblasts are highly
125 sensitive to EtOH during terminal differentiation.

126

127 **3.2 Junctional zone cell markers**

128 EtOH exposure caused dose-dependent decreases in gene expression of *Tpbpa* (Fig1.B), a
129 marker of both fully differentiated junctional zone trophoblasts and their earlier ecto-
130 placental cone progenitors [23]. Additionally, both *Prl7a2,* a marker of spongiotrophoblast
131 (SpT) and parietal trophoblast giant cells (P-TGCs), and *Prl7b1* a marker of invasive
132 glycogen trophoblast (GlyT) cells and spiral artery-associated TGCs (SpA-TGCs) were
133 decreased by EtOH exposure (Fig.1C,D). Curiously, this suggestion of reduced GlyTs *in vitro*
134 conflicts with the observation of increased area occupied by this cell type in the junctional
135 zone of periconceptionally exposed placentas *in vivo* [6]. It is tempting to speculate that
136 altered GlyT differentiation may also have affected their invasive behaviour as seen in other
137 EtOH exposure models [13]. No differences were found in the 0.2% EtOH groups for *Prl7b1*
138 or *Prl7a2* relative to control or 1% groups.

139 No alterations were found for *Prl3d1* (*Pl1*, Fig.1.E), or *Prl2c (Plf,* Fig.1.F) which are
140 expressed by secondary TGCs *in vivo*, indicating that EtOH exposure does not alter

ACCEPTED MANUSCRIPT

141 expression of all trophoblast subtype specific gene markers. When this same experiment was
142 performed on another cell line – RS26, similar expression profiles were seen at day 6 for all
143 junctional zone trophoblast markers with the exception of *Tpbpa*, which did not change
144 (*Supp. Fig.2A-F*). This difference between the TS lines may be either due to clonal
145 differences between cell lines, or alternately a sex-specific result, as the RS26 line is male
146 and the EGFP line is female (data not shown). Maternal perturbations commonly result in
147 sexually dimorphic impacts on placental structure, function, and differentiation - evidenced
148 by GlyT cell accumulation in the junctional zone of females only following periconceptional
149 EtOH exposure [6]. Further examination of multiple male and female cell lines would be
150 required to determine this possibility.

151 The trophoblast subtype markers in this study have been used previously to infer effects of
152 differentiation of specific trophoblast subtypes. However, it is also possible that EtOH may
153 be directly altering gene expression levels, rather than the differentiation of the trophoblast
154 subtypes per se. Therefore to confirm whether EtOH reduces the number of differentiated
155 trophoblast subtypes, FISH was carried out on cells collected from day 6 cultures to examine
156 the number of *Prl7a2* and *Tpbpa* positively labelled cells. Each marker was first localised in
157 paraffin sections of mouse placentas to confirm reported trophoblast subtype specific
158 labelling (Fig.2.A,D). Quantification of *Prl7a2* positive (Fig 2.B,C,G) and *Tpbpa* positive
159 (Fig 2.E,F,H) trophoblasts *in vitro* showed no difference between treatments although there
160 was considerable variability in the cells cultured in 1% EtOH. This suggests that exposure to
161 EtOH did not overtly affect the number of differentiated trophoblasts of each subtype, but
162 may indicate either less maturity of trophoblasts or more simply, reduced expression of the
163 genes themselves. Considering the heterogeneous cell populations marked by these genes, it
164 is possible that subtle differences in number of trophoblast subtypes may still be apparent.

165 **3.3 Labyrinth zone cell markers**

ACCEPTED MANUSCRIPT

166 Finally we examined the impact of EtOH on expression of labyrinthine-specific trophoblast
167 markers. While no alteration was found in *Ctsq* (Fig.2G) expression, representing sinusoidal
168 TGCs (S-TGCs), EtOH exposure did cause a dose-dependent decrease in gene expression for
169 *Syna* (Fig.1H), marking syncytiotrophoblast layer 1 (SynT-I). *Syna* expression was
170 significantly decreased in the 1% group relative to the control whilst *Syna* expression in 0.2%
171 EtOH exposed cells was intermediate and not significantly different to control or 1% EtOH
172 groups. Exposure of the RS26 line to EtOH also revealed reduced *Syna* expression in the 1%
173 EtOH group, also with no changes to *Ctsq* (*Supp.Fig.1F,G*). This demonstrates that EtOH has
174 the potential to affect trophoblast viability and differentiation in multiple placental
175 compartments. Furthermore, these observations indicate that a commonly used vehicle for
176 dissolving drugs in itself has a significant impact on trophoblast behaviour, and must be
177 properly controlled for *in vitro* experiments.

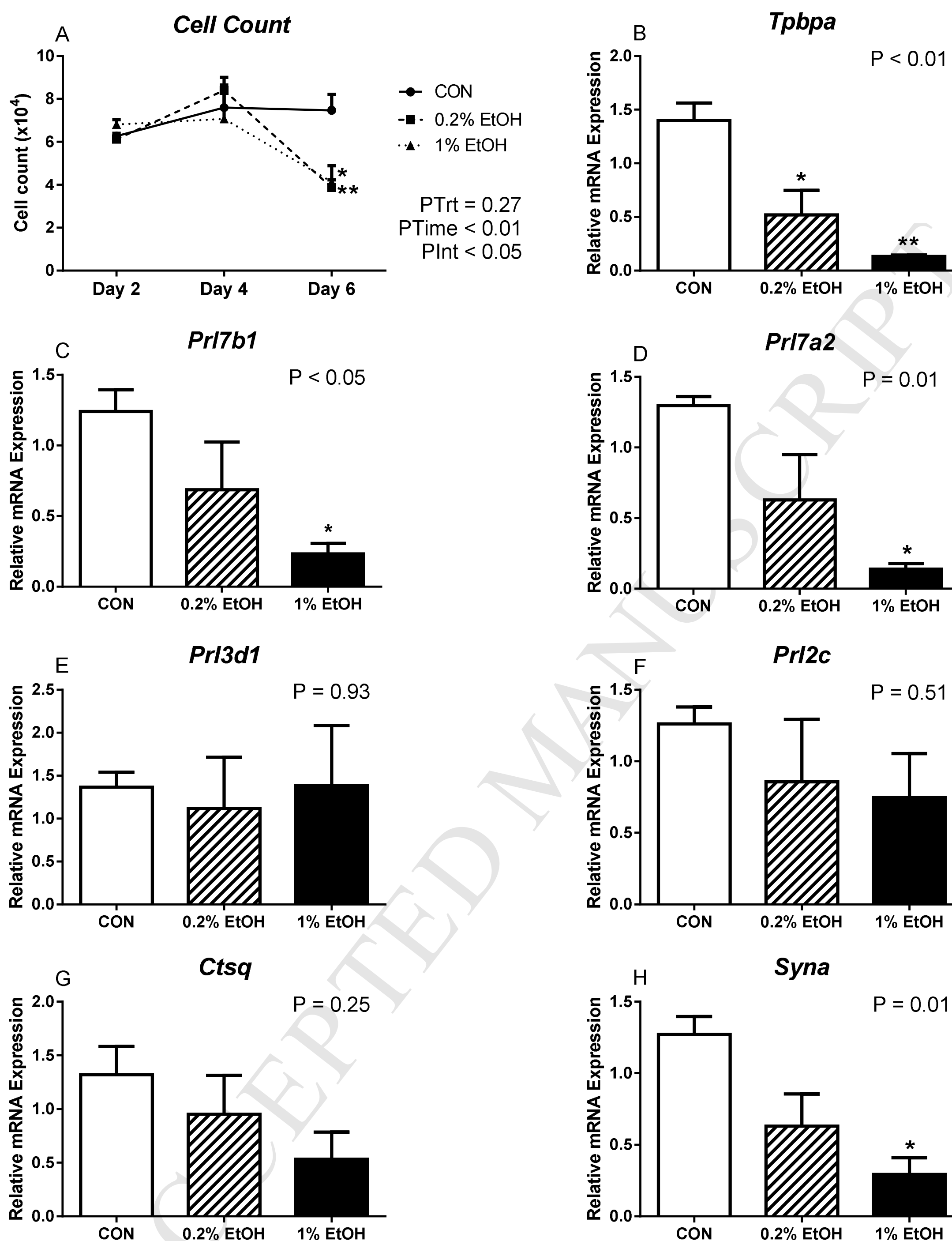
178 **3.4 Conclusions**

179 This study demonstrates that EtOH exposure can directly alter the viability of mature
180 trophoblasts and the expression of trophoblast subtype markers for both labyrinth and
181 junctional zones. Alterations to labyrinthine cell types may alter barrier thickness or labyrinth
182 architecture potentially impacting appropriate nutrient exchange, whilst changes to invasive
183 GlyT and SpA-TGCs may alter their invasive behaviour and lead to poor invasion. Future
184 studies on how alterations of these cell types *in vivo* may lead to altered placental structure
185 and function is now paramount in determining the impacts of EtOH exposure.

186

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT



187
188
189 **Fig.1. Trophoblast stem cell proliferation and expression profiles when exposed to EtOH over 6**
190 **days during differentiation.** Panel A. Proliferation analyses showing control (circle), 0.2% EtOH
191 (square), 1% EtOH (triangle), *N*=3/treatment. Panels B-H show gene expression profiles of
192 trophoblast subtype markers for junctional zone (B-F) and the labyrinth (G and H), with control
193 (white bars), 0.2% EtOH (striped bars), 1% EtOH (black bars). All data analysed by one-way
194 ANOVA with Tukey's post-hoc tests. *N*=9/treatment. All data represented by mean ± SEM. *P<0.05,
195 **P<0.01 compared to CON.
196

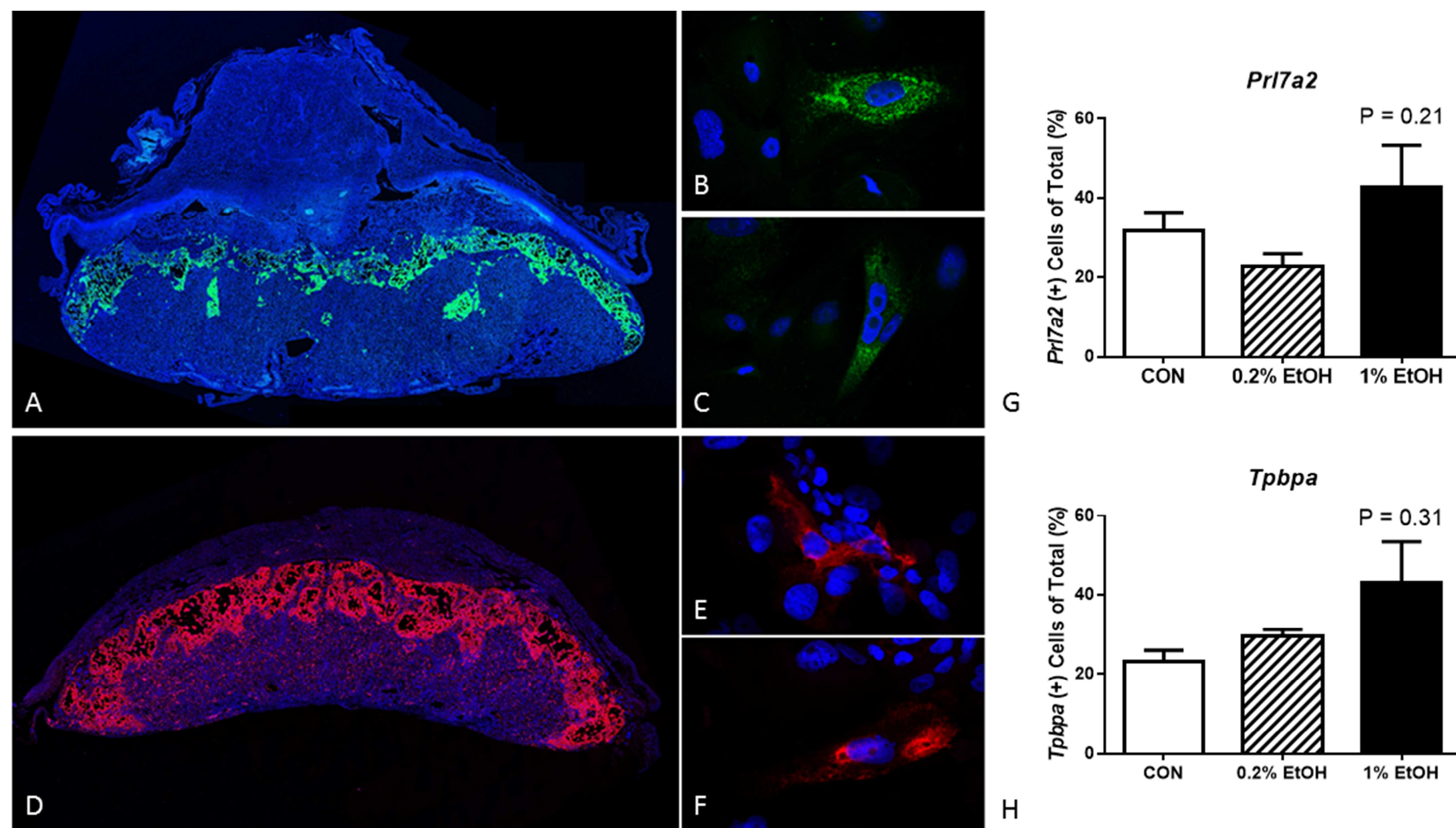


Fig.2. Quantification of *Prl7a2 and Tpbpa* positive trophoblasts by fluorescent *in situ* hybridisation. Panels A-C show representative images of *Prl7a2* (green) expression in SpT and P-TGCs in the E12.5 mouse placenta at low (10x) (A) magnification. B and C show representative images of EGFP cells cultured to day 6. Panel G shows percentage *Prl7a2* positive cell counts of total cultured cells at day 6 (*N*=3/trt). Panels D-F show representative images of *Tpbpa* (red) expression in SpT, GlyT, and junctional zone progenitors in the E14.5 placenta mouse placenta at low (D) magnification. E and F show representative images of cells cultured to day 6. Panel H shows percentage *Tpbpa* positive cell counts of total cultured cells at day 6 (*N*=3/trt). All nuclei stained with Hoechst (blue). All data represented by mean ± SEM. Data analysed by one-way ANOVA with Tukey's or Kruskal-Wallis post-hoc test.

ACCEPTED MANUSCRIPT

Acknowledgments

JIK and JEO are recipients of Australian Postgraduate Award scholarships. KMM is a Senior Research Fellow of the NHMRC. This study was supported by a grant from the National Health & Medical Research Council of Australia.

Statement of Interest
None.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

[1] KWONG, W. Y., WILD, A. E., ROBERTS, P., WILLIS, A. C. & FLEMING, T. P. 2000. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development,* 127, 4195-202.

[2] KWONG, W. Y., MILLER, D. J., URSELL, E., WILD, A. E., WILKINS, A. P., OSMOND, C., ANTHONY, F. W. & FLEMING, T. P. 2006. Imprinted gene expression in the rat embryo-fetal axis is altered in response to periconceptional maternal low protein diet. *Reproduction,* 132, 265-77.

[3] KWONG, W. Y., MILLER, D. J., WILKINS, A. P., DEAR, M. S., WRIGHT, J. N., OSMOND, C., ZHANG, J. & FLEMING, T. P. 2007. Maternal low protein diet restricted to the preimplantation period induces a gender-specific change on hepatic gene expression in rat fetuses. *Mol Reprod Dev,* 74, 48-56.

[4] EDWARDS, L. J. & MCMILLEN, I. C. 2002a. Impact of maternal undernutrition during the periconceptional period, fetal number, and fetal sex on the development of the hypothalamo-pituitary adrenal axis in sheep during late gestation. *Biol Reprod,* 66, 1562-9.

[5] MACLAUGHLIN, S. M., WALKER, S. K., ROBERTS, C. T., KLEEMANN, D. O. & MCMILLEN, I. C. 2005. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *J Physiol,* 565**,** 111-24.

[6] GARDEBJER, E. M., CUFFE, J. S., PANTALEON, M., WLODEK, M. E. & MORITZ, K. M. 2014. Periconceptional alcohol consumption causes fetal growth restriction and increases glycogen accumulation in the late gestation rat placenta. *Placenta*.

[7] GARDEBJER, E. M., ANDERSON, S. T., PANTALEON, M., WLODEK, M. E. & MORITZ, K. M. 2015. Maternal alcohol intake around the time of conception causes glucose intolerance and insulin insensitivity in rat offspring, which is exacerbated by a postnatal high-fat diet. *FASEB J*.

[8] SIMMONS, D. G., FORTIER, A. L. & CROSS, J. C. 2007. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. *Dev Biol,* 304, 567-78.

[9] CROSS, J. C., WERB, Z. & FISHER, S. J. 1994. Implantation and the placenta: key pieces of the development puzzle. *Science,* 266, 1508-18.

[10] COAN, P. M., FERGUSON-SMITH, A. C. & BURTON, G. J. 2004. Developmental dynamics of the definitive mouse placenta assessed by stereology. *Biol Reprod,* 70, 1806-13.

[11] COLVIN, L., PAYNE, J., PARSONS, D., KURINCZUK, J. J. & BOWER, C. 2007. Alcohol consumption during pregnancy in nonindigenous west Australian women. *Alcohol Clin Exp Res,* 31, 276-84.

[12] WALLACE, C., BURNS, L., GILMOUR, S. & HUTCHINSON, D. 2007. Substance use, psychological distress and violence among pregnant and breastfeeding Australian women. *Aust N Z J Public Health,* 31, 51-6.

[13] GUNDOGAN, F., GILLIGAN, J., OOI, J., SUNG, J., QI, W., NARAM, R. & DE LA MONTE, S. M. 2013. Dual Mechanisms of Ethanol-Impaired Placentation: Experimental Model. *J Clin Exp Pathol,* 3.

[14] GUNDOGAN, F., GILLIGAN, J., QI, W., CHEN, E., NARAM, R. & DE LA MONTE, S. M. 2015. Dose effect of gestational ethanol exposure on placentation and fetal growth. *Placenta*.

ACCEPTED MANUSCRIPT

[15] HIMENO, E., TANAKA, S. & KUNATH, T. 2008. Isolation and manipulation of mouse trophoblast stem cells. *Curr Protoc Stem Cell Biol,* Chapter 1, Unit 1E 4.

[16] LUI, S., JONES, R. L., ROBINSON, N. J., GREENWOOD, S. L., APLIN, J. D. & TOWER, C. L. 2014. Detrimental effects of ethanol and its metabolite acetaldehyde, on first trimester human placental cell turnover and function. *PLoS One,* 9, e87328.

[17] MORITZ, K. M., JOHNSON, K., DOUGLAS-DENTON, R., WINTOUR, E. M. & DODIC, M. 2002. Maternal glucocorticoid treatment programs alterations in the renin-angiotensin system of the ovine fetal kidney. *Endocrinology,* 143, 4455-63.

[18] RODRIGUEZ, F. D., SIMONSSON, P. & ALLING, C. 1992. A method for maintaining constant ethanol concentrations in cell culture media. *Alcohol Alcohol,* 27**,** 309-13.

[19] WATSON, E. D., HUGHES, M., SIMMONS, D. G., NATALE, D. R., SUTHERLAND, A. E. & CROSS, J. C. 2011. Cell-cell adhesion defects in Mrj mutant trophoblast cells are associated with failure to pattern the chorion during early placental development. *Dev Dyn,* 240, 2505-19

[20] SIMMONS, D. G., NATALE, D. R., BEGAY, V., HUGHES, M., LEUTZ, A. & CROSS, J. C. 2008. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. *Development,* 135, 2083-91.

[21] CLAVE, S., JOYA, X., SALAT-BATLLE, J., GARCIA-ALGAR, O. & VALL, O. 2014. Ethanol cytotoxic effect on trophoblast cells. *Toxicol Lett,* 225, 216-21.

[22] BOLNICK, J. M., KARANA, R., CHIANG, P. J., KILBURN, B. A., ROMERO, R., DIAMOND, M. P., SMITH, S. M. & ARMANT, D. R. 2014. Apoptosis of Alcohol-Exposed Human Placental Cytotrophoblast Cells is Downstream of Intracellular Calcium Signaling. *Alcohol Clin Exp Res,* 38, 1646-53.

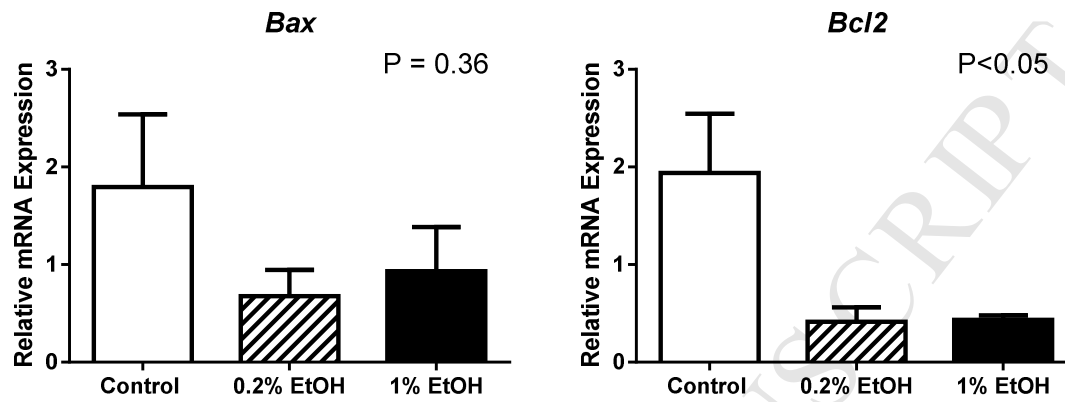
[23] ADAMSON, S. L., LU, Y., WHITELEY, K. J., HOLMYARD, D., HEMBERGER, M., PFARRER, C. & CROSS, J. C. 2002. Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol,* 250**,** 358-73.

[24] CHAN, W. H. & CHANG, Y. J. 2006. Dosage effects of resveratrol on ethanol-induced cell death in the human K562 cell line. *Toxicol Lett,* 161**,** 1-9.

ACCEPTED MANUSCRIPT

Supplementary Figures.

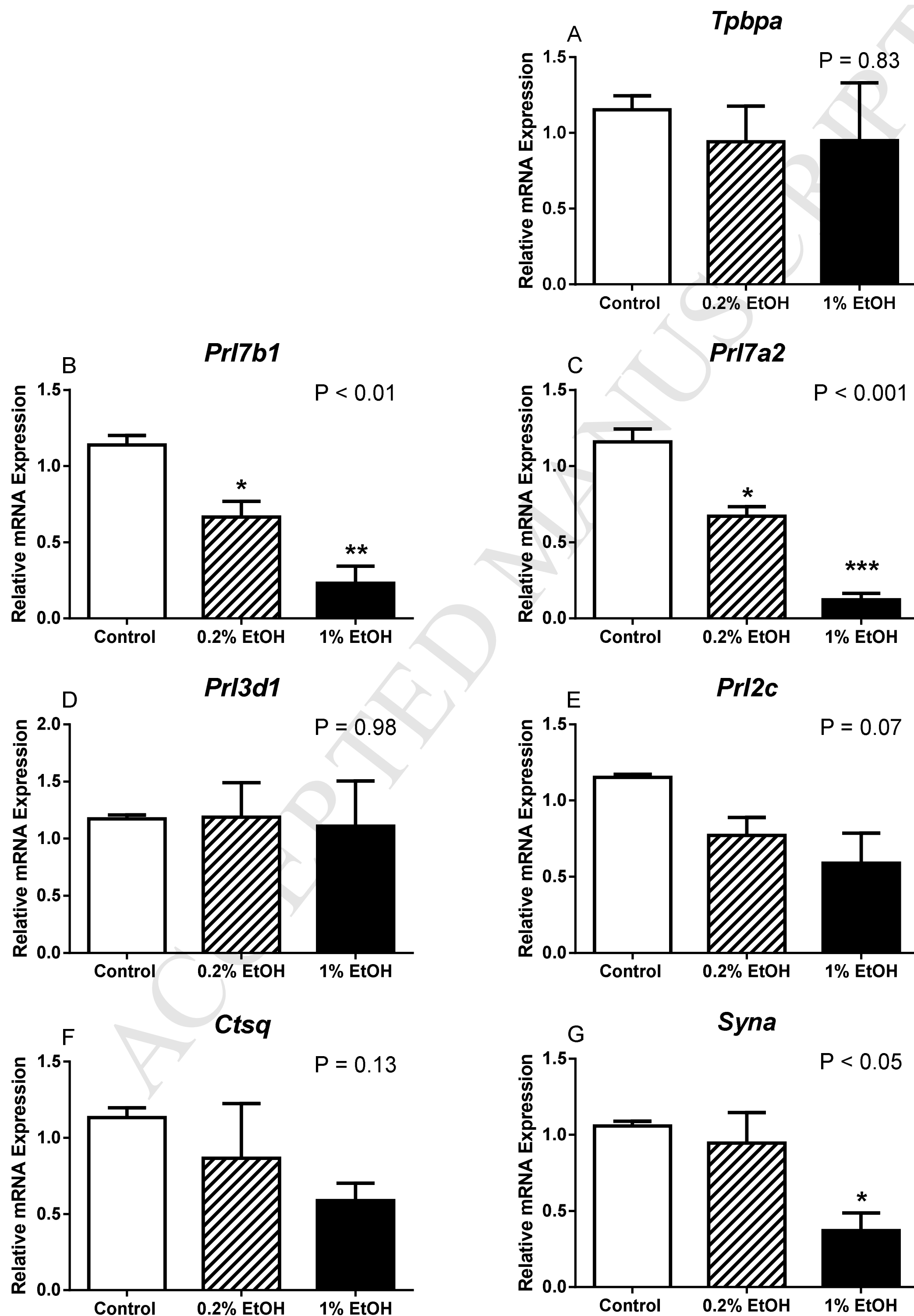
Supp. Figure 1. Gene expression of pro-apoptotic marker Bax and anti-apoptotic marker Bcl2



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Supp. Figure 2. Expression profiles of RS26 differentiated trophoblasts at day 6 after EtOH exposure. Panels A-G show gene expression profiles of trophoblast subtype markers for junctional zone (A-E) and the labyrinth (F and G), with control (white bars), 0.2% EtOH (striped bars), 1% EtOH (black bars). RS26 line was seeded at 10,000 cells per well to control for proliferative capacity. All data analysed by one-way ANOVA with Tukey's post-hoc tests. *N*=9/treatment. All data represented by mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to CON.



ACCEPTED MANUSCRIPT

Supplementary Materials and Methods

Table 1. Mouse trophoblast markers and associated primer sequences for SYBR qRT-PCR.

Gene	Trophoblast Marker	Forward Primer	Reverse Primer
Junctional Zone			
Prl7a2	SpT, P-TGC	GGGAGAATGTGGCCTCTGTA	TTGAGCTTCGTCCAGGTTTT
Prl7b1	GlyT/SpA-TGC	GGGAGGACGTGGTCTCTGTA	TTTGGTGATTTGAGTGGCAA
Tpbpa	JZ and progenitors	AAGTTAGGCAACGAGCGAAA	AGTGCAGGATCCCACTTGTC
Prl3d1 (Pl1)	1° P-TGC	CTGCTGACATTAAGGGCA	AACAAAGACCATGTGGGC
Prl2c (Plf)	2° TGCs	TGTGTGCAATGAGGAATGGT	TAGTGTGTGAGCCTGGCTTG
Labyrinth			
Syna	SynT-1	ATGGAGAAACCCCTTACGCT	TAGGGGTCTTTGTGTCCCTG
Ctsq	S-TGC	TTCATTGGCCCAATACCCTA	GAAAGCTCCCAGAATTCACA
Other			
18S	Housekeeper	GTAACCCGTTGAACCCATT	CCATCGAATCGGTAGTAGCG

TS – trophoblast stem cell, SpT – spongiotrophoblast, GlyT – glycogen trophoblast, SpA-TGC – spiral artery-associated trophoblast giant cell, JZ – junctional zone, P-TGC – parietal trophoblast giant cell, TGC – trophoblast giant cell, SynT-1/2 – syncytiotrophoblast layer 1/2, S-TGC – sinusoidal trophoblast giant cell, 18S – ribosomal RNA.

Pro- and anti-apoptotic marker analysis by qPCR

TaqMAN gene expression assays utilised 5ul TaqMAN (Qiagen), *Bax* Mm00432051, *Bcl2* Mm00477631, and *18S* 4319413E (Thermofisher). *Bax* and *Bcl2* were multiplexed to *18S.*

Fluorescent in situ hybridisation for localisation of trophoblast subtypes on cultured EGFP cells

ISH for TS cell cultures were carried out as described [8], with modifications for fluorescent localisation. Fluorescein or DIG-labelled probes were made for *Tpbpa* and *Prl7a2* as per manufacturer's instructions (Roche) with primers sequences as described previously [8,20]. In brief, cells were fixed in 4% PFA for 15 min and transferred to 0.4% PFA until analysis. Cells were not treated with proteinase K, but were acetylated for 5 minutes prior to administration of FITC- or DIG-labelled probes at 1:200 which were hybridised overnight at 65°C. The following day, cells were washed in 5xSSC pre-warmed to 65°C for 3x20 min, followed by TN buffer (100mM Tris-HCl ph7.5, 150mM NaCl) for 5 mins prior to incubation

ACCEPTED MANUSCRIPT

in block consisting of 1× MABT (150 mM NaCl, 100 mM maleic acid, 0.1% Tween-20, pH 7.5), 2% blocking reagent (Roche), 20% heat inactivated horse serum, for 1h. Samples were incubated in anti-DIG-POD or anti-FITC-POD antibody (1:500, Roche) overnight. The following day, cells were washed in TNT buffer containing 0.05% Tween20 in TN buffer for 3x15 min prior to probe detection with 1:50 FITC- or Cy5-Tyramide in amplification reagent (TSA^{TM} kit, Perkin Elmer) for 10 min. Samples were washed in TNT buffer for 3x5 min and counterstained with Hoechst (Sigma). Coverslips were mounted in 70% glycerol prior to imaging. Prior to use on cultured cells, probes were tested on either E12.5 or E14.5 mouse placentas fixed in 4% paraformaldehyde overnight.

Imaging and Analysis

Coverslips were imaged in 20x20 frames in z stacks of 5 using a 20x air objective on an inverted microscope (Leica) using set parameters. *Prl7a2*-FITC was imaged with the 488nm laser, *Tpbpa*-DIG was imaged with the 561nm laser and Hoechst with the 405nm laser. Representative images were also taken of paraffin and cultured cells using either the 20x or 40x air objectives. Image analysis was carried out using ImageJ (NIH). Z stacks were compressed with the maximum projections for DAPI and merged (DAPI and FISH marker). The greyscale DAPI image was converted to 8 bit, underwent watershed to separate adjoining nuclei, and counted with the automated Analyse Particles function. FISH positive cells were counted from merged images using Cell Counter.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Highlights:

- Trophoblast cell survival in vitro was inhibited by ethanol
- Ethanol reduced gene expression of labyrinth and junctional zone trophoblast subtype-specific markers
- Results suggest ethanol exposure can directly alter the viability of mature trophoblasts and the expression of trophoblast subtype markers for both labyrinth and junctional zones

ACCEPTED MANUSCRIPT