Expression of aldehyde dehydrogenase family 1, member A3 in glycogen trophoblast cells of the murine placenta

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Title: Expression of aldehyde dehydrogenase family 1, member A3 in glycogen trophoblast cells of the murine placenta

Short title: Aldh1a3 expression by murine glycogen trophoblast cells

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Conflicts of Interest
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Abstract

Introduction: Retinoic acid (RA) signaling is a well known regulator of trophoblast differentiation and placental development, and maternal decidual cells are recognized as the source of much of this RA. We explored possible trophoblast-derived sources of RA by examining the expression of RA synthesis enzymes in the developing mouse placenta, as well as addressed potential sites of RA action by examining the ontogeny of gene expression for other RA metabolizing and receptor genes. Furthermore, we investigated the effects of endogenous RA production on trophoblast differentiation.

Methods: Placental tissues were examined by in situ hybridization and assayed for RARE-LacZ transgene activity to locate sites of RAR signalling. Trophoblast stem cell cultures were differentiated in the presence of ALDH1 inhibitors (DEAB and citral), and expression of labyrinth (Syna, Ctsq) and junctional zone (Tpbpa, Prl7b1, Prl7a2) marker genes were analysed by qRT-PCR.

Results: We show Aldh1a3 is strongly expressed in a subset of ectoplacental cone cells and in glycogen trophoblast cells of the definitive murine placenta. Most trophoblast subtypes of the placenta express RA receptor combinations that would enable them to respond to RA signaling. Furthermore, expression of junctional zone markers decrease in differentiating trophoblast cultures when endogenous ALDH1 enzymes are inhibited.

Discussion: Aldh1a3 is a novel marker for glycogen trophoblast cells and their precursors and may play a role in the differentiation of junctional zone cell types via production of a local source of RA.
1. Introduction

In mice, the process of placentation requires the expansion of a
trophectoderm-derived stem cell population into specialized trophoblast cells
with unique behaviours. Early in development an intermediary pool of
progenitors, the ectoplacental cone (EPC), give rise to cells of the definitive
junctional zone (JZ) comprising glycogen trophoblast (GlyT) and
spongiotrophoblast (SpT) cells plus additional subtypes of trophoblast giant
cells (TGCs) (1,2). GlyT and SpT cells of the JZ form the intermediate layer of
the definitive placenta and are thought to play endocrine and structural roles,
whereas TGCs, which differ in subtype depending on their vascular location,
manipulate and direct maternal blood supply while likewise contributing to
placental hormone function (1-3). In contrast, cells of the chorion give rise to
trophoblast subtypes of the labyrinth zone, the inner layer of the placenta
involved in mediating fetal-maternal exchange.

RA signaling is known to regulate key aspects of placental development and
function. Mice deficient in retinoid X receptors (RXRα or β) or peroxisome
proliferator activated receptors (PPARβ/δ or γ) have severe placental
phenotypes (4-7). Additionally, deficiencies in maternal vitamin A result in
placental failure in rodents (8,9), and in vitro studies have shown the
involvement of retinoids in the upregulation of pregnancy specific hormones
produced by trophoblast cells (10,11) and the regulation of trophoblast
differentiation (12,13) and invasion (14,15).

Aldehyde dehydrogenase family 1, member A3 (ALDH1A3) belongs to a large
gene family of enzymes known to metabolise a wide variety of aldehydes
ALDH1A3 specifically oxidizes retinal to retinoic acid (RA) and is differentially expressed in developing embryonic tissues and adult organs (15-17). The RA produced by ALDH1A3 in rodents contributes to the development of skin and hair follicles, brain, tooth buds, lungs, olfactory bulbs, kidneys, eyes, skeletal muscle and seminal vesicles (17-19).

The source of RA acting on trophoblast cells during placentation is thought to be primarily from maternal decidual cells (13,20). In the present study, we report that a subset of cells within the EPC and later, GlyT cells of the definitive murine placenta, express Aldh1a3. This suggests that GlyT are a local source of RA during placental development. Furthermore, we explored what possible role trophoblast-derived RA may be playing in the placenta by analyzing the expression patterns of retinoic acid metabolizing enzymes and receptors in the developing placenta, as well as by differentiating trophoblast stem cells in the absence of endogenous RA production.

2. Methods

2.1 Animals and tissue preparation

C57BL6 mice were used for all in situ hybridisation (ISH) and Best's Carmine staining procedures. Recognition of a seminal plug was designated embryonic day (E) 0.5. Whole implantation sites (E8.5-E10.5) or placentas (E12.5-E18.5) were dissected in cold phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde (PFA) at 4°C and processed for paraffin embedding.
RARE-LacZ homozygous transgenic male mice maintained on a CD1 background (kindly provided by Peter Koopman, IMB) were crossed with wild type CD1 females for detection of retinoic acid response element (RARE) activity. Samples were fixed in 2% PFA/0.2% glutaraldehyde solution in 1x PBS for 4 hours at 4°C before freezing in OCT-media (Tissue Tek), as previously described (1). Animals were housed in accordance with the University of Queensland animal facility guidelines, and the University of Queensland Animal Ethics Committee approved all experiments.

2.2 Histology

β-galactosidase detection was performed on 10 µm OCT sections as previously described (1). Seven µm paraffin sections were used for Best’s Carmine staining as previously described (21).

2.3 In Situ Hybridisation

Genes targeted for in situ hybridization (ISH) analysis are listed in Supplemental Table 1. cDNA probe templates were isolated by gel extraction (Qiagen Minelute Gel Extraction Kit) or via “PCR clean up” (Qiagen Minelute PCR purification) and sequence verified by the Australian Genome Research Facility (AGRF). Digoxigenin (DIG) labeled cRNA probes were synthesized in accordance with manufacturer’s directions (Roche) and as previously described (22). In situ hybridizations on 7µm paraffin-embedded sections were done as previously described (23). Negative controls included the use
of sense probes for all mRNA analyzed, and positive controls included the
detection of antisense probes in positive control tissues.

2.4 Trophoblast stem cell culture

Trophoblast stem (TS) cells were cultured as previously described (24).
Differentiation of TS cells was achieved by replacing conditioned media with
TS media alone (without FGF4 or heparin) in the presence of either citral
(30µM), diethylaminobenzaldehyde (DEAB; 100µM) or DMSO (vehicle
control).

2.5 RNA isolation, cDNA production and qRT-PCR

RNA was isolated from placental tissue and TS cell cultures using TRIzol
reagent (Life Technologies) following the manufacturer’s instructions. Total
RNA (1µg) was used to generate cDNA (Qiagen Quantitect cDNA synthesis
kit) according to the manufacturer’s instructions. mRNA expression levels
were assessed by quantitative real-time PCR (qRT-PCR). Primer sequences
are listed in Supplemental Table 2. Each SYBR green qRT-PCR reaction
contained 5µL SYBR green (ABI), 1µL of cDNA (150 ng/µL) plus 4µM primers
in a final volume of 10µL. Relative cDNA levels were analyzed in triplicate for
each independent experimental replicate (n=3). Differences between
expression levels were determined by the Pfaffl method of qRT-PCR analysis
with normalization to Rn18s, Hprt1 and Rpl13a gene expression (25).
2.6 Statistics

To determine statistically significant differences (* p<0.05; ** p<0.005) the mean relative level of expression plus standard deviations were calculated for all qRT-PCR results and unpaired, two-tailed t-tests were performed. All graphs and statistics were performed using GraphPad Prism 5 software.

3. Results

3.1 Aldh1a3 expression is restricted to a subset of ectoplacental cone cells and glycogen trophoblast cells of the definitive mouse placenta.

Expression of Aldh1a3 mRNA was analyzed spatially and temporarily by in situ hybridisation (ISH) in mouse implantation sites and placental sections from E8.5 to E18.5 (Fig 1A/B, Fig S1/S2). Midline coronal sections of implantation sites at E8.5 show Aldh1a3 expression in some decidual cells and within a subpopulation of EPC trophoblast cells (Fig 1A). Expression patterns for Pcdh12 and Aldh1a3 within the developing EPC match, while expression of Prl7b1 and Aldh1a3 do not entirely overlap (Fig S3, Fig 1G/H).

By E10.5 expression of Aldh1a3 is seen within a subset of cells situated in the JZ, which by E12.5-E18.5 were also observed clustered within the maternal decidua (Fig 1B, Fig S1). Aldh1a3 positive cells were confirmed to be GlyT based on their distinct cell morphology and by comparison with serial sections stained with Best’s Carmine to detect glycogen content (Fig 1C/D), Prl7b1 (Fig 1E/F) and Pcdh12 expression (Fig S3). Expression of Aldh1a3 in uterine decidual cells was not observed from E10.5 to E18.5 (Fig 1B, Fig S1).
GlyT cells form a layer around luminal cells of the maternal blood spaces, a layer known to include SpA-TGC (lining spiral arteries) and C-TGCs (lining the central canals) (26). Serial coronal sections comparing Aldh1a3 and Prl7b1 expression at E14.5 nicely recapitulate this phenomenon (Fig 1I/J). Aldh1a3 and Prl7b1 expression marks GlyT cells in the maternal decidua, adjacent to SpA-TGCs that express Prl7b1 only (Fig 1J). Interestingly, around mid-gestation GlyT cells immediately adjacent to the maternal spiral arteries appear to lose their Aldh1a3 expression (Fig 1I).

3.2 Expression of retinoic acid metabolizing enzymes within the mouse placenta.

Messenger RNA expression patterns for RA metabolizing enzymes Aldh1a1 and Aldh1a2 plus RA catabolizing enzymes Cyp26a1, Cyp26b1 and Cyp26c1 were analyzed by ISH. Aldh1a2 was expressed at the extreme mesometrial pole of the E8.5 uterus, as well as within the embryo proper, although no expression was seen in the developing EPC or chorion (Fig 2A). By E14.5, strong expression of Aldh1a2 was observed throughout the entire maternal decidual compartment of the mature placenta (Fig 2B). Cyp26b1 expression was observed in the uterus at the anti-mesometrial pole and in the P-TGC layer surrounding the conceptus at E8.5 (Fig 2C/D), while expression was detected sporadically amongst clusters of GlyT cells within the JZ by E14.5 (Fig 2E). Expression of Aldh1a1, Cyp26a1 or Cyp26c1 was not observed in the placenta at either developmental stage (Fig S4).
3.3 Placental sites of potential RA action.

To determine which placental cell types may be capable of responding to GlyT-derived RA, analysis of retinoid receptor (Rara/b/g and Rxra/b/g) expression was analyzed at E8.5 and E14.5. Non-classical RA signaling was also examined through ISH analysis of the alternate RXR binding partners the Ppar isoforms (Ppard/g). For brevity ISH results are summarised in Figure 3 (individual ISH images have been included in supplemental information; Fig S5-S12 and a detailed description in Supplemental file 1). Briefly, most placental trophoblast subtypes, along with maternal decidual cells, appear to express some combination of RA receptors. In theory, this expression should allow these cells to respond to RA signaling.

RARE-LacZ reporter mice (27) were used to assess the presence of classical RA signaling through RARs during placental development. While robust LacZ staining was observed in the developing E8.5 embryo, no expression of the LacZ transgene could be detected in the EPC or chorion (Fig 4A). Punctate LacZ expression was observed in a small subset of cells dispersed throughout the labyrinth and JZ at E14.5 (Fig 4B/C).

3.4 Inhibition of endogenous ALDH activity alters trophoblast stem cell differentiation.
Citral and DEAB (acting as non-competitive and competitive ALDH1 substrates, respectively) were employed to inhibit ALDH1 activity, and therefore the synthesis of endogenous RA, in the trophoblast stem (TS) cell model (28). TS cells differentiated over six days in the presence of citral, DEAB or DMSO vehicle were analysed by qRT-PCR for changes in expression of several trophoblast cell type markers. Labyrinth cell type specific genes *Syna* (syncytiotrophoblast layer-I), *Ctsq* (S-TGCs), and P-TGC marker *Prl3d1/PL1* showed no significant change in mRNA expression following the addition of either citral or DEAB. In contrast, JZ cell type markers *Tpbpa* (EPC, SpT and GlyT), *Prl7a2* (SpT specific) and *Prl7b1* (GlyT and SpA/C-TGC specific) were expressed at significantly lower levels after six days of differentiation in the presence of citral. Similar trends were observed in the presence of DEAB, with the reduction of *Tpbpa* and *Prl7b1* statistically significant (Fig 5, Fig S13). *Aldh1a3* expression in differentiating TS cells was confirmed by qRT-PCR (Fig S14); surprisingly we also found that TS cells expressed *Aldh1a2* (data not shown), which is not expressed by trophoblast but by decidual cells *in vivo*.

**Discussion**

ALDH1A3, along with other ALDH1 family members (ALDH1A1 and A2), generate RA (15,16). Recently *Aldh1a3* has been recognized as a marker of various stem cell populations, including cancer stem cells, and is suggested to play a role in the homeostasis of these progenitors (29-31). In the current study we demonstrate expression of *Aldh1a3* first in a subset of EPC
progenitor cells, and then within GlyTs of the definitive placenta. While gene
expression patterns do not prove cell lineage, the tight restriction of Aldh1a3
expression to GlyT cells of the definitive placenta strongly suggests that
earlier expression within the EPC marks GlyT progenitors. GlyT were
originally thought to arise from SpT of the JZ, until Bouillot et al. demonstrated
Pcdh12 expression in a subset of central EPC cells at E7.5, followed by
restricted expression in GlyT but not SpT cells of the JZ (32). Recent cell
lineage tracing experiments support this notion; SpA-TGCs, C-TGCs and
GlyT, but not SpT cells, are derived from Prdm1 positive EPC progenitors
(33). We analysed Aldh1a3 and Prl7b1 expression in E8.5 serial sections.
Prl7b1 expression is restricted to the Prdm1 lineage, first expressed by subset
of EPC cells and then by SpA-TGCs, C-TGCs and GlyT of the definitive
placenta (22). We observed cells positive for Prl7b1 but negative for Aldh1a3
near the top of the EPC, consistent with the location of SpA-TGCs, as well as
cells with overlapping Aldh1a3 and Prl7b1 expression within the core of the
EPC, consistent with the location of future GlyT. Interestingly, we also
observed Aldh1a3+/Prl7b1− cells at the base of the EPC, suggesting Aldh1a3
expression may be an earlier marker of GlyT cell progenitors than Prl7b1.
Lineage tracing experiments using an Aldh1a3-driven Cre recombinase-
expressing transgenic mouse line would illuminate the early developmental
origins of the SpA-TGC, C-TGC and GlyT lineages within the developing
EPC.

It has been suggested that the RA required for placentation is maternally
derived (13,20). Expression of Aldh1a3 by a subset of EPC progenitors, and
subsequently by GlyT cells of the JZ, suggests an additional source of placental RA. RA produced by EPC/GlyT cells would be positioned to influence the differentiation or maintenance of most placental cell types, but JZ subtypes in particular.

Altered RA signalling has previously been shown to perturb placental development; the dearth of Rar expression in trophoblast cells, the lack of placental phenotypes in Rar mutant mice (34), and the absence of LacZ staining in RARE-LacZ placentas reiterate that non-classical RA pathways are likely responsible. Retinoid X receptors are well placed in the developing and mature placenta to propagate 9-cis-RA signaling, and mouse knockout studies have highlighted their fundamental importance, possible redundancy, and potential for homodimerisation. Rxra\(^{-/-}\) and Rxra\(^{-/-}\)/Rxrb\(^{-/-}\) double null mutants die in mid gestation due to a number of placental defects including decreased GlyT cells, reduced JZ and disorganized labyrinth development (4,5).

Ppard and Pparg null mutants similarly reveal key roles for these RXR binding partners in placentation. Unlike RXRs, PPARs are noted for their non-redundant roles in placentation. The loss of Ppard \textit{in vivo} leads to smaller placentas with limited decidual contact, often resulting in placental detachment and lethality by E10.5 (7). In line with this we note that all Rxrs and Ppard are expressed in the P-TGC layer. Alternatively, Pparg knockout mice exhibit placental failure at E9.5 reportedly due to decreased differentiation of terminal trophoblast cell types (6). The expression of Pparg is
strong in most trophoblast cells, with the exception of P-TGCs, and remains
so for the duration of placentation. The role of PPARγ/RXRα heterodimers in
placental biology has been extensively investigated and studies implicate
pleiotropic functions in trophoblast development and maintenance (35). RA
synthesized by GlyT is likely to influence several trophoblast cell types in the
developing placenta, predominantly through PPAR and RXR signaling.

P-TGCs are potentially exposed to both decidual-derived RA (via ALDH1A2)
as well as GlyT-derived RA (via ALDH1A3), and all-trans RA induces
trophoblast differentiation towards a P-TGC fate both in vivo and in vitro
(1,13). Interestingly, P-TGCs express Cyp26b1 at E8.5, an enzyme that
catabolizes active RA and protect cells from its differentiating effect (36).
Cyp26b1 expression may coincide with terminally differentiated P-TGCs no
longer requiring RA signaling. Alternatively, one could speculate that
Cyp26b1 expression may act to sensitize P-TGCs to 9-cis-RA signaling;
CYP26A1 and B1 have a specificity for catabolizing all-trans-RA, whereas
CYP26C1, of which we found no expression in trophoblast cells, preferentially
catabolizes 9-cis-RA (37). Cyp26b1 expression by P-TGCs could possibly
exclude all-trans-RA signaling following differentiation while retaining
responsiveness to 9-cis-RA signaling, presumably through RXR/PPARβ/δ.
Such a mechanism could distinguish between different types of RA signaling,
perhaps segregating differentiation signals from those required for cell
behaviours such as migration and endocrine production.
Cyp26b1 expression was also detected in an unidentified JZ cell type. The expression of Cyp26b1 in this environment may identify JZ progenitor cells that would be needed for the ongoing expansion of this layer or for secondary-TGCs, as Cyp26b1 expression would protect these cells from the differentiating effects of RA. It has previously been suggested that JZ cells are able to terminally differentiate into secondary TGC as O2 increases near vessels (38). As we observed a loss of Aldh1a3 in GlyT cells closest to spiral arteries, it is tempting to speculate that this may allow differentiation into SpA-TGCs as spiral arteries expand in the second half of gestation. This notion may also help to explain the discrepancy observed between GlyT cell numbers leaving the JZ and arriving in the MD (39), if they are being recruited to form SpA-TGCs.

Inhibition of endogenous ALDH1 activity led to a significant decrease in JZ cell type markers Tpbpa, Prl7b1 and Prl7a2 by citral and a significant reduction in Tpbpa and Prl7b1 expression by DEAB in TS cell cultures. While our in vitro TS cell experiments cannot inform us as to the function of GlyT-derived RA later in gestation, they do indicate that differentiating TS cells are influenced by endogenously produced RA. The decrease in Tpbpa, Prl7b1 and Prl7a2 expression following ALDH1 inhibition highlights a potential function of EPC ALDH1A3 in regulating differentiation and expansion of the JZ trophoblast population.

Expression of Aldh1a3 in mature GlyT cells of the definitive placenta may play an autocrine role, facilitating proliferation and driving invasion into the
maternal decidua. Following E12.5, GlyT increase greatly in number and begin invading interstitially into the decidua in streams which remain in contact with one another (39). PPARγ/RXRα heterodimers, both of which are expressed in GlyT, have been implicated in the invasion of human extravillous cytotrophoblast, an analogous cell type to murine GlyT (14,40). Aldh1a3 is also the predominant isoform in breast cancer stem cells and is a robust predictor of metastatic behaviour (41).

While the function of GlyT cells remains incompletely understood, Aldh1a3 expression in the early EPC suggests GlyT progenitors may play a role in regulating the early differentiation of JZ cell types, a notion supported by inhibition of endogenously produced RA in differentiating TS cell cultures. Later expression of Aldh1a3 in GlyT suggests a role in GlyT functions such as migration, or even perhaps in the differentiation into later born SpA-TGCs. It would be informative to analyze the placentae of Aldh1a3 mutant embryos, which die shortly after birth due to defects in airway development, to ascertain whether differentiation of JZ cell types, GlyT invasion or spiral artery remodeling is adversely affected, and therefore whether there is a placental contribution to the developmental phenotypes seen in mutant embryos.

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Author Contributions

J.E.O. performed and interpreted most experiments, B.V.N performed some in situ hybridizations, D.G.S. and D.R.C.N. conceived of the project, J.E.O. and D.G.S. wrote the manuscript with input from all authors.

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Figure Legends

Figure 1. Aldh1a3 expression is restricted to cells of the ectoplacental cone (EPC) and glycogen trophoblast (GlyT) cells of the definitive mouse placenta.

A: Aldh1a3 expression is seen in cells of the maternal decidua above the uterine lumen (UL) and in trophoblast cells of the EPC at E8.5. B: Aldh1a3 mRNA is detected in cells of the junctional zone (JZ) at E14.5. C/D: Serial sections show analogous expression of Aldh1a3 mRNA and Best’s Carmine glycogen staining in GlyT cells of the JZ at E12.5, respectively. Higher magnification depicted in boxed areas. E/F: Serial sections show comparable expression patterns for Aldh1a3 (E) and Prl7b1 (F) at E14.5 in GlyT cells throughout the JZ. G/H: Serial sections at E8.5 show expression of Aldh1a3 (G) and Prl7b1 (H) in the ectoplacental cone (EPC). While these markers can be seen highlighting the same population of cells, Aldh1a3 (G) also appears to be solely marking a discrete population in the centre of the EPC (dashed outline). Note that Prl7b1 (H) expression, though stronger, appears to also mark an increased number of peripheral EPC cells. I/J: Serial sections at E14.5 of the maternal decidua show glycogen trophoblast cells appear to lose their expression of Aldh1a3 (I) when situated close to maternal spiral arteries (MSAs), indicated by arrows. Prl7b1 (J) expression on the other hand is not lost in these cells. In addition, Prl7b1 is seen to mark spiral artery-associated trophoblast giant cells of the MSAs, indicated by red asterisks. Ch, chorion; Dec, decidua; Lab, labyrinth; SpT, spongiotrophoblast cells. Scale Bars = 1mm (red), 100µm (black).
Figure 2. Expression of retinoic acid metabolising enzymes Aldh1a2 and Cyp26b1 in the mouse placenta. **A:** Expression of Aldh1a2 can be seen in the mesometrial pole of the uterus and in the embryo (E) proper at E8.5. **B:** At E14.5 Aldh1a2 is restricted to the maternal decidua (Dec). **C/D:** At E8.5 Cyp26b1 expression is seen in the parietal-TGC layer (C) and anti-mesometrial pole (D) of the uterus. **E:** At E14.5 Cyp26b1 can be seen expressed throughout the junctional zone (JZ) in an unidentified cell type. Lab, labyrinth. Scale bars = 1mm (red), 100µm (black).

Figure 3. Overview of In situ hybridisation (ISH) results for mRNA expression of Rar (a/b/g), Rxr (a/b/g) and Ppar (d/g) in coronal placental sections at E8.5 (A) and E14.5 (B). (+) Reported in this study; for details refer to supplemental file 1; ISH images provided in supplemental figures (Fig S4 to Fig S11). [1] Sapin et al, 1997 (42); [2] Nadra et al, 2006 (43); [3] Barak et al, 1999 (7).

Figure 4. Classical retinoic acid signalling is seen in the labyrinth (Lab) and junctional zone (JZ) of the definitive mouse placenta. **A:** At E8.5 coronal cryosections through the implantation site are negative for LacZ expression in the developing chorion, ectoplacental cone (EPC) or parietal-TGC layer. LacZ positive cells are evident in the embryo (E) proper. **B/C:** LacZ positive cells can be seen in unknown cell types of the JZ (B) and Lab (C) at E14.5. Scale bars = 1mm (red), 200µm (black).

Figure 5. ALDH inhibitors (Citral/DEAB) downregulate the expression of junctional zone cell type markers in differentiated (Day 6) trophoblast stem
(TS) cell cultures. **A-C:** Labyrinth cell type specific markers (Prl3d1, Syna, Ctsq) show no significant change in expression following the loss of endogenous ALDH1 activity. **D-F:** Junctional Zone cell type markers (Tpbpa, Prl7a2, Prl7b1) show significantly less expression in differentiated TS cells following treatment with Citral. **D:** Tpbpa and Prl7b1 were also significantly decreased following treatment with DEAB (* p<0.05, ** p<0.005).
Supplemental Figure Legends

**Figure S1:** In Situ Hybridization showing *Aldh1a3* spatial expression from E8.5 to E18.5 in coronal placental sections. **A:** Implantation site at E8.5 shows *Aldh1a3* expression in the expanding ectoplacental cone (EPC). Expression can also be seen throughout the maternal decidua above the uterine lumen (UL), E: Embryo. **B:** E10.5 placental section shows expression of *Aldh1a3* in the expanding junctional zone (JZ) adjacent to the developing labyrinth (Lab). Note expression of *Aldh1a3* in the decidua is lost. **C:** E12.5 placenta shows expression of *Aldh1a3* in glycogen trophoblast (GlyT) cells of the JZ. **D:** E14.5 placenta sees *Aldh1a3* mRNA expressed in GlyT cells within the JZ and GlyT cells migrating into the decidua in close proximity to the maternal spiral arteries. *Aldh1a3* transcripts can also be seen in GlyT cells at E16.5 (E) and E18.5 (F) within the JZ and maternal decidua. Higher magnifications shown in boxed areas.

**Figure S2:** In Situ Hybridization showing spatial expression of *Prl7b1* (A-F) compared to *Aldh1a3* (A1-F1) in transverse serial sections of E8.5 ectoplacental cone (EPC). **A:** The uterine lumen (UL) can be seen central to each section. There are no *Prl7b1* (A) positive cells. *Aldh1a3* (A1) expression is evident in the maternal decidua. **B:** Cells of the EPC begin to emerge and are positive for both *Prl7b1* (B) and *Aldh1a3* (B1). *Aldh1a3* positive maternal stromal cells are also present in the periphery (B1). **C/D:** Central EPC cells are positive for both *Aldh1a3* (C1/D1) and *Prl7b1* (C/D). **E/F:** Expression of *Prl7b1* (E/F) mRNA is lost. A small number of *Aldh1a3* (E1/F1) positive cells are still detected as sections approach the chorion (Ch) of the developing placenta. Cells expressing *Aldh1a3* can be seen for a further 140 microns beyond the loss of expression of *Prl7b1*. Cartoon inset shows the orientation of UL, EPC and developing Ch at E8.5. Dashed lines represent the level of *Prl7b1* (black) and *Aldh1a3* (blue) sections.

**Figure S3:** In Situ Hybridization showing expression of *Aldh1a3* (A/C) matches that of *Pcdh12* (B/D) in serial sections of the ectoplacental cone at E8.5 (A/B), and in glycogen trophoblast cells within the junctional zone and maternal decidua at E14.5 (C/D).

**Figure S4:** In Situ Hybridization depicting lack of expression of various retinoic acid metabolising enzymes in mouse placental tissue. **A/B:** *Aldh1a1* is not expressed in placental tissue at E8.5 or E14.5. **C:** Expression of *Aldh1a1* can be seen in the lens of the adult mouse eye, used as control tissue. **D:** *Cyp26a1* expression is absent from E8.5 ectoplacental cone and chorion, however can be seen in uterine epithelium shown in higher magnification in boxed area. **E:** *Cyp26a1* is also absent from E14.5 placental tissue, however expression is evident in uterine epithelial tissue shown in higher magnification in boxed area. **F/G:** *Cyp26c1* is also absent from E8.5 and E14.5 placental sections. Expression is evident in the developing embryo at E8.5 (F).

**Figure S5:** **A/B:** Expression ofRARα is absent from the ectoplacental cone (EPC) and majority of the chorion at E8.5 (A). **RARα** mRNA is weakly evident in the allantois (A) and in the base of the chorion (B). **C:** Expression of **RARα** is seen throughout the mesometrial pole of the uterus and in cells lining uterine glands at E8.5. **D:** Expression of **RARα** in the labyrinth at E14.5 is detected weakly throughout with stronger expression associated with fetal vasculature.
Figure S6: A/B: Expression of RARβ mRNA is not evident in placental tissue at E8.5 or E14.5.

Figure S7: A/B: Expression of RARγ is absent from the ectoplacental cone (EPC) and majority of the chorion at E8.5. RARγ mRNA is weakly evident in the expanding allantois, where it joins the base of the chorion and at branching points at this time. C: At E14.5 expression of RARγ is seen diffusely throughout the maternal decidua. D: Expression of RARγ in the labyrinth at E14.5 is most strongly associated with the fetal vasculature.

Figure S8: A/B: In situ hybridisation analysis of RXRα mRNA shows expression in the ectoplacental cone (EPC), chorion (Ch) and parietal trophoblast giant cell (asterisk) layer at E8.5. C/D: E14.5 expression of RXRα mRNA. D: Higher magnification shows transcripts present in the labyrinth (beneath dotted line) and in clusters of glycogen trophoblast cells (arrows) throughout the junctional zone and maternal decidua. RXRα is not seen in spongiotrophoblast cells of the junctional zone.

Figure S9: A: Expression of RXRβ mRNA at E8.5 can be seen in the ectoplacental cone (EPC) and chorion (Ch). B/C: Sections at E14.5 show RXRβ mRNA present in the labyrinth (beneath dotted line) and in clusters of glycogen trophoblast cells (arrows) of the junctional zone and maternal decidua, seen more clearly in higher magnification (C). Some weak expression may also be present in spongiotrophoblast cells.

Figure S10: A/B: In situ hybridisation analysis of RXRγ mRNA shows expression in the chorion (Ch) and parietal trophoblast giant cell (arrows) layer at E8.5. Strong expression is also evident in cells of the ante-mesometrial pole of the uterus (B). C/D: E14.5 expression of RXRγ mRNA. D: Higher magnification shows weak expression of transcripts in the labyrinth (beneath dotted line) and in clusters of glycogen trophoblast cells (asterisk) throughout the junctional zone and maternal decidua. RXRγ is not seen in spongiotrophoblast cells of the junctional zone.

Figure S11: A: In situ hybridisation analysis of PPARδ/β reveals expression throughout the ectoplacental cone and in the top layer of cells of the chorion at E8.5 (seen in higher magnification in boxed area). B: PPARδ/β mRNA is seen in parietal trophoblast giant cells surrounding the implantation site at E8.5. C: Weak expression of PPARδ/β can be seen throughout the junctional zone and labyrinth in E12.5 placental sections. Expression of PPARδ/β mRNA in glycogen trophoblast cells (asterisk) of the junctional zone at E12.5 can be seen in higher magnification (boxed area).

Figure S12: A: In situ hybridisation analysis of PPARγ shows expression throughout the ectoplacental cone and in the chorion at E8.5. PPARγ mRNA is absent from the parietal trophoblast giant cell layer (asterisk) at this time point. B-D: PPARγ mRNA expression in E14.5 coronal placental sections. C: Expression of PPARγ can be seen throughout the junctional zone in glycogen trophoblast cells and spongiotrophoblast cells at E14.5. D: Expression of PPARγ mRNA in cells of the labyrinth at E14.5.

Figure S13: Additional qRT-PCR results for expression levels of various trophoblast cell marker genes in ALDH1 inhibitor treated trophoblast stem (TS) cells normalised to Hprt1 and Rpl13a house keeper genes. As with Rn18s, downregulation of expression of
junctional zone cell type markers in differentiated (Day 6) TS cell cultures was observed. **A-C:** Labyrinth cell type specific markers (*Prl3d1* and *Ctsq*) show no significant change in expression following the loss of endogenous ALDH1 activity. *Syna* shows a decrease in expression in citral treated cells when normalised to *Hrpt1* expression. **D-F:** Junctional Zone cell type markers (*Tpbpa, Prl7b1, Prl7a2*) show significantly less expression in differentiated TS cells following treatment with Citral. **D/E:** *Tpbpa* and *Prl7b1* were also significantly decreased following treatment with DEAB (*p<0.05, **p<0.005, ***p<0.001).

**Figure S14:** Quantitative RT-PCR results of endogenous *Aldh1a3* expression in trophoblast stem cell cultures over 6 days of differentiation.
Highlights

- *Aldh1a3* is expressed by glycogen trophoblast cells of the murine placenta
- Glycogen trophoblast cells may be a local source of placental retinoic acid
- All trophoblast subtypes of the mouse placenta express some complement of RA receptors
- Inhibition of endogenous ALDH1 enzymes in TS cell cultures alters differentiation
Detailed description of ISH analysis of RA metabolizing enzymes and receptors during murine placental Development.

At E8.5, Rara transcripts were expressed within the mesometrial pole and glandular epithelium of the uterus (Fig 3, Fig S4C), plus weak and diffuse expression of Rarg in the mesometrial and anti-mesometrial poles of the uterus, consistent with previously reported findings (1) (data not shown). Rara and Rarg were also found to be weakly expressed in the allantois with Rara present in the base of the chorion (Fig S4A/B, S6A/B). No expression of Rarb was observed (Fig S5A). Expression of all Rxr isoforms was seen in both poles of the uterus (data not shown), the EPC and developing chorion (Fig S7A/S8A/S9A) plus the P-TGC layer (Fig S7B/S8A/S9B) surrounding the implantation site. Interestingly, chorionic expression of Ppard was restricted to the top layer of the chorion (Fig S10A), and was also seen in the EPC and P-TGC layer surrounding the conceptus (Fig S10B). In contrast, Pparg was excluded from the P-TGC layer but was evident within the EPC and chorion (Fig S11A). No uterine expression of either Ppar isoform was observed.

At E14.5 Rara and Rarg were expressed in cells associated with the fetal derived vasculature within the labyrinth (Fig S4D/S6D), while Rarg also exhibited diffuse expression throughout the maternal decidua (Fig S4C). No expression of Rarb was detected in the definitive placenta (Fig S5B). Expression of Rxra was observed weakly throughout the maternal decidua (MD) (data not shown) and more definitively in GlyT and labyrinth cell types (Fig S7D). Similarly, Rxrg was expressed in GlyT of the JZ and cells of the labyrinth (Fig S9D). Rxrb transcripts were detected in the labyrinth, GlyT and possibly a small subset of SpT cells of the JZ (Fig S8C). The Ppard isoform was detected weakly at E12.5 in JZ cell types and additionally in the expanding labyrinth (Fig S10C). Of note, Ppard expression appeared to decrease following a peak at E10.5 and was undetected by E16.5 (data not shown), consistent with previously reported relative mRNA expression findings (2). Pparg was observed in GlyT and SpT cells of the JZ and extensively throughout the labyrinth (Fig S11B-D).
β-galactosidase activity, while robust in the developing embryo at E8.5, was not seen in the EPC or chorion of the developing placenta. This is consistent with receptor mRNA expression, as we found no evidence of overlapping $Rar/Rxr$ expression in the developing EPC at E8.5. At E14.5, while sparse, lacZ expression was observed in areas that had some expression of both $Rar$ and $Rxr$ transcripts.

References


**Table 1:** Primer pair sequences used in the production of In Situ Hybridisation probes.

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Probe templates were generated by PCR with included RNA polymerase binding sites (T3 – Forward and T7 – Reverse as indicated; RNA polymerase binding sites are represented in bold). * Some probe templates were cloned into pGEM-Teasy and therefore lacked T3 or T7 sequences in the primers. The forward and reverse probes for Prl7b1 have been previously described (1).

Table 2: Primer pair sequences for individual genes used in qRT-PCR

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Rn18s: 18s Ribosomal RNA, Aldh1a3: Aldehyde dehydrogenase 1 family, member a3, Prl3d1: Prolactin family 3, subfamily d, member 1, PL1: Placental lactogen 1, Syna: syncytin a, Ctsq: Cathepsin Q, Tpbpa: Trophoblast specific protein a, Prl7b1: Prolactin family 7, subfamily b, member 1, Prl7a2: Prolactin family 7, subfamily a, member 2.