Atypical Protein Kinase C iota in trophoblast lineage development and establishment of the maternal-fetal interface

By

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Abstract

Mammalian embryonic development depends on the indispensable contribution of a transient dynamic organ, the placenta, which supports the pregnancy during the entire gestational days. The placenta provides hormones to regulate fetal development, act as the nutrient exchange surface and as a protective barrier for the fetus during its most crucial initial days inside the mother's uterus. Impaired development of such a crucial organ will imperatively lead to pregnancy associated disorders including preterm birth, intrauterine growth retardation (IUGR), preeclampsia and even the severe form of eclampsia. A lot of research studies are now focusing on the disruptive effects of placental disorders, most of which are molecular signaling anomalies leading to defective trophoblast lineage development. Also, many of the embryonic lethal mouse mutants have been found to be associated with placental insufficiency not reported before, which is the most vital underlying molecular cause for embryonic death. In humans, atleast 40% of all pregnancies undergo spontaneous miscarriages and the etiology for these cases and others like recurrent pregnancy losses (RPLs) remains unknown. For ethical reasons studying human tissues and subjects remain tightly regulated. Alternatively, rodents, which also exhibit hemochorial placentation similar to humans, have been used as a powerful model to study the molecular mechanisms important for placentation. In this study, we have identified one of the key conserved signaling mechanism which we believe is essential for the proper development of the placenta both in rodents as well as in humans. Earlier research have shown the importance of Protein Kinase C (PKC) isoform, Atypical PKC iota (PKCλ/ι) in embryonic stem (ES) cells and mitochondrial biogenesis in self-renewing versus differentiating ES cells, however the functional importance of PKCλ/ι in the trophoblast lineage development remained oblivious despite of embryonic lethal phenotype upon global deletion of the gene coding PKCλ/ι, *Prkci* in mouse. In this study, we have

used the global knockout mouse model of *Prkci* gene and we show that PKCλ/ι is essential for the establishment of the maternal-fetal exchange surface made up of trophoblast cell subtype called the syncytiotrophoblasts (SynTs). Our analyses using mouse and human trophoblast stem (TS) cells indicate the essentiality of PKCλ/ι in establishing the differentiation program in trophoblast progenitors towards SynTs. Our whole genomic analyses, using PKCλ/ι depleted mouse and human TS cells, reveal that known regulators of SynT development such as GATA-binding factor 2 (GATA2) and Peroxisome proliferator-activated receptor gamma (PPARG), act downstream of PKCλ/ι signaling. GATA2 and PPARG in turn regulates GCM1/DLX3 signaling axis. Both GATA2 and PPARG undergoes significant downregulation in protein and mRNA expression thus impairing SynT establishment in PKCλ/ι depleted mouse and human TS cells. In the second part of this study, we have analyzed a conditional knockout mouse model of *Prkci*, where it is deleted using a Cre-mediated recombination, specifically, in the Syncytiotrophoblast II (SynT-II) cells of mouse placenta. Our results so far indicate that homozygous deletion of *Prkci* allele in the SynT-II cells causes developmental defects amounting to embryonic lethality in these mutants. Summarizing the whole study, we have identified novel, evolutionary conserved functional importance of Atypical Protein Kinase C isoform PKCλ/ι in inducing differentiation program within trophoblast progenitors towards the establishment of the maternal–fetal exchange surface from mouse to humans.

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Chapter 1: Introduction

The appearance of the placenta at the feto-maternal junction happened in the eutherian mammals some thousands of years back. Since then the placenta acts as the transport surface where maternal blood bathes the fetal compartment. It also provides the exchange interface for nutrients and metabolites. The placenta not only functions as the exchange interface but also provides an immunosuppressive milieu for the semi-allogeneic fetus to survive and develop. Both human and rodents show hemochorial placentation, where the maternal blood comes into direct contact with the trophoblast derived tissues.

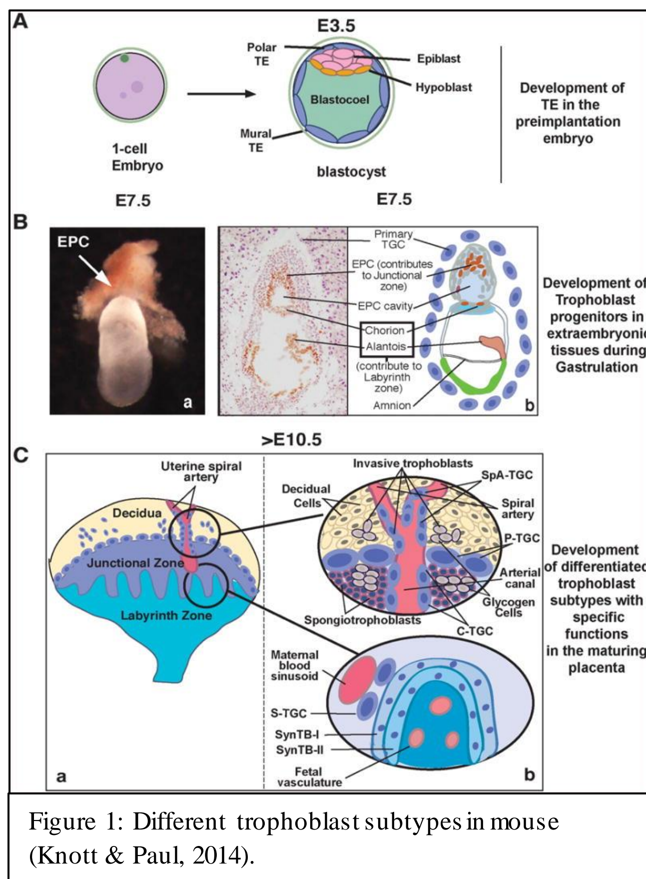

Figure 1: Different trophoblast subtypes in mouse (Knott & Paul, 2014).

Mouse placenta development:

The establishment of the trophoblast lineage in mouse takes place as early as embryonic day (E) 3.5 post fertilization. The fertilized zygote undergoes rapid cell division and gives rise to a ball of around 200 cells known as the blastocyst. This is the first stage of lineage specification taking place in mouse embryonic development where the cells are either destined to become part of the trophectoderm (TE), giving rise to the entire extraembryonic lineage or inner cell mass (ICM), giving rise to the entire embryonic compartment. The TE also encloses the blastocoelic cavity by a monolayer of tight junction-coupled epithelium with the ICM cells at one end. During the 8 to 16 cell division stage, the cells acquire apical protein domains selectively and undergoes

polarity-induced internalization (Anani, Bhat, Honma-Yamanaka, Krawchuk, & Yamanaka, 2014; Johnson & Ziomek, 1981; Watanabe, Biggins, Tannan, & Srinivas, 2014). Inner cells remain apolar whereas the outside cells remain either polar or apolar. This polarity difference between outer and inner cells results in differential Hippo signaling activation. The polar outside cells with the inactivated Hippo signaling leads to Yap protein translocating to the nucleus along with its co-factor Tead4, turning on Cdx2 expression, sealing the trophectoderm (TE) fate (Hirate et al., 2013; Nishioka et al., 2009; Rayon et al., 2014). Cdx2 activation in the TE leads to downregulation of pluripotency factors like Oct4 and Nanog (L. Chen et al., 2009; Niwa et al., 2005; Dan Strumpf et al., 2005) whereas activated Hippo signaling in the ICM leads to upregulation of Sox2, another pluripotency factor (Wicklow et al., 2014). Recent studies using Cdx2-eGFP as a readout for Hippo signaling, ICM cells were found to retain plasticity till the early blastocyst (64 cell) stage whereas TE cells became restricted much earlier in the 32 cell stage (Posfai et al., 2017). Cdx2 expressing trophoblast cells maintain their self-renewing property and undergoes proliferation under the influence of FGF, secreted from the inner cell mass. The ICM cells will eventually give rise to 2 lineages- Nanog expressing epiblast and Gata6 expressing primitive endoderm (PE) (Katie Cockburn & Janet Rossant, 2010).

After lineage segregation into TE and ICM, the blastocyst undergoes implantation within the maternal endometrium in mouse at around (E) 4-4.5. The trophectoderm cells in close proximity to the ICM called polar TE, continues to proliferate under the influence of fibroblast growth factor receptor ligand, Fgf4 (Rappolee, Basilico, Patel, & Werb, 1994), whereas the trophectoderm cells further away from the ICM, start to differentiate giving rise to primary trophoblast giant cells (TGCs). Eventually the polar TE cells differentiate in a second wave of differentiation giving rise

to the ectoplacental cone (EPC) and the 'intermediate' extraembryonic ectoderm (ExE), which will eventually form the chorion at around (E) 6.5-7.

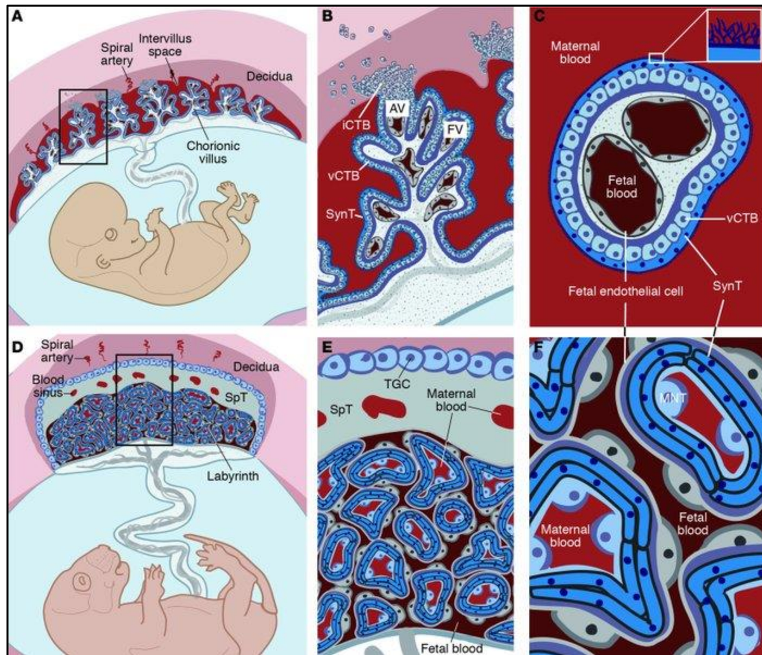

Figure 2: Comparison between the structure of mouse and human placenta (Maltepe, Bakardjiev, & Fisher, 2010).

Within the EPC, there continues further lineage segregation with differential expression of trophoblast specific protein alpha (Tpbpa). *Tpbpa* positive cell clusters differentiate and gives rise to the junctional zone (JZ) or the spongiotrophoblast. However, the Tpbpa negative cells of the basal EPC start to express Distal less 3 (Dlx3) and contribute to the SynTI layer of the mouse syncytiotrophoblast.*Tpbpa* negative cells also differentiate towards the sinusoidal trophoblast giant cells (S-TGC), which comes into direct contact with maternal blood sinuses and start to express cathepsin (Ctsq) at around (E) 8.5 (David G. Simmons et al., 2008).

The labyrinthine vascular tree of the placenta initiates when the allantoic mesoderm extends and fuses with the basal chorionic cells at around (E) 8-8.5 and is referred to as the chorio-allantoic attachment. This fusion event is mediated by cell adhesion molecule, Vcam1, present at the tip of allantois (Gurtner et al., 1995) and integrin alpha 4 (Itga4) (J. T. Yang, Rayburn, & Hynes, 1995) at the basal surface of the chorion. This event marks the initiation of glial cells missing 1 (Gcm1) expression at the focal points of invagination or branching morphogenesis for labyrinth development (Anson-Cartwright et al., 2000a; Cross, Nakano, Natale, Simmons, & Watson, 2006;

David G. Simmons et al., 2008). Gcm1, which was first identified in Drosophila melanogaster as essential for neuronal differentiation, starts to express in the chorion as early as (E) 8. Gcm1 expressing cells at the tip of elongating branches are separated by 4-6 non-expressing cells, which express Reproductive homeobox 4B (Rhox4b). Gcm1 deficient mice fail to undergo labyrinth morphogenesis, the chorion persists as a flat tissue without branching and invagination by fetal derived blood vessels (Anson-Cartwright et al., 2000a; Schreiber, Riethmacher-Sonnenberg, Riethmacher, Tuerk, Enderich, Bösl, et al., 2000). Gcm1 continues to express as long as the villus branching continues to expand (Basyuk et al., 1999b). Gcm1 gives rise to the SynT-II layer of the trilaminar trophoblast of the placental labyrinth. After the establishment of the labyrinth, the placenta expands in size and provides more surface area for nutrient exchange to support the growing fetus.

Human placenta development:

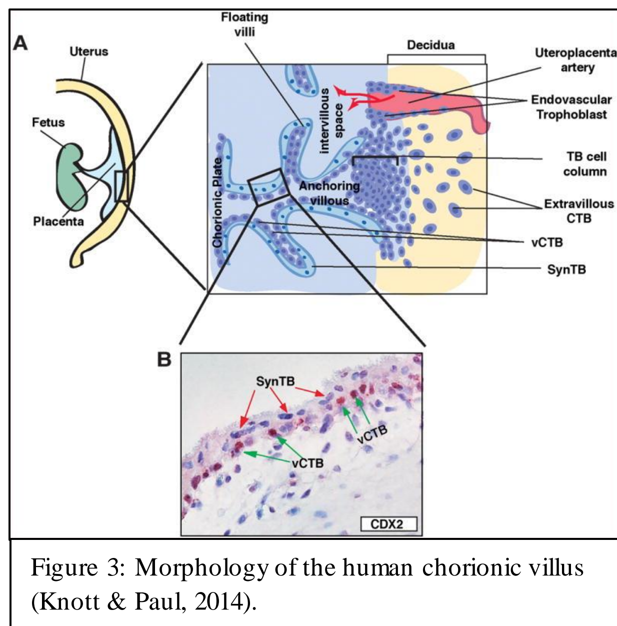

Figure 3: Morphology of the human chorionic villus (Knott & Paul, 2014).

The human placenta is hemochorial, similar to rodents. However, understanding the anatomic, physiologic and endocrinologic difference across gestation stages which last about 36-39 weeks has a challenge. The actual picture of the human utero-placental and intervillous circulation is a matter of debate since several hundred years, as researchers have made use of methodologies at their disposal and often the results obtained reflected the use of that particular research tool.

One of the earliest consistent anatomic evidence of the structures of the human placenta in the first three months of pregnancy came from Hamilton and Boyd (Hamilton & Boyd, 1960) where they acquired in situ specimens across different staged embryos starting from 11 days after fertilization to the stage where the embryo was 60mm in size. The specimens were obtained through a series of collaborations between numerous pathologists and obstetricians who collected specimens for several years. Hamilton and Boyd classified these various gestational stages into the Barnes embryo (11-12 day stage), the Missen embryo (14-18 day stage), the Gar embryo, the Shaw embryo, embryos in somite stages (20-30days), embryos of 10mm stage (37-38days), embryos at 15mm stage (41-43 days), embryos between 20-30mm size (48-60days) and embryos between 31-60 mm size (60-90days). Each of these stages have been well defined by the authors with photographs depicting the anatomical details.

During the first 12 weeks of pregnancy, referred to as the first trimester, the uteroplacental arteries are plugged by trophoblast cells prohibiting blood flow between the definitive placenta and the maternal circulation, instead the intervillous space is bathed by acellular fluid supposed to be plasma which is filtered by the trophoblastic shell (Hustin & Schaaps, 1987). These trophoblastic plugs dislocate by week 11-12 initiating blood flow into the intervillous space. The Doppler ultrasonography has been an instrumental tool in studying the hemodynamics of blood flow in the uteroplacental arteries. Other in vivo studies utilizing the Doppler ultrasonography have also confirmed absence of uteroplacental blood flow during the first trimester of gestation (Jaffe & Warsof, 1991; Jaffe & Woods, 1993; Jauniaux, Jurkovic, & Campbell, 1991; Jauniaux, Jurkovic, Campbell, & Hustin, 1992). Measurement of oxygen tension within the placental bed also corroborates with the previous mentioned studies. This prevention of uterine blood flow is to protect the developing fetus from mechanical and biochemical stresses imposed by the higher

velocity flow in the spiral arteries. At this phase, the developing fetus acquires nutrition from the uterine glandular secretions and this mode of nutrition is termed as histiotrophic nutrition. Evidences showing phagocytic uptake of mucin (MUC-1) and glycodelinA, which are synthesized within the glands, by the syncytiotrophoblast indicates histiotrophic mode of nutrition uptake in the first few weeks of gestation (Burton, Watson, Hempstock, Skepper, & Jauniaux, 2002). Eventually, after the establishment of blood circulation, the switch from histiotrophic to hemotrophic mode of nutrition takes place. Oxygen tension in the placenta during the transition from first to second trimester (13-26 weeks of pregnancy), increases from <20mm Hg to >50mm Hg. This rise of oxygen tension also initiates increased mRNA synthesis of antioxidant enzymes, rearrangement of the cristae within mitochondria of the syncytiotrophoblast and upregulation of heat shock protein 70 which indicates a burst in oxidative stress as blood circulation is established (Jauniaux et al., 2000; Rodesch, Simon, Donner, & Jauniaux, 1992).

The functional and structural unit of a human placenta is the chorionic villi. Each of these chorionic villi are made up of progenitor cells called cytotrophoblast which fuse to form the outer syncytiotrophoblast. The cytotrophoblast cells residing within the villus are termed as villus cytotrophoblast (vCTB) or cell column cytotrophoblast (cCTB). However, the cCTBs towards the distal end of the anchoring villus differentiates and give rise to the third type of trophoblasts, the invasive trophoblasts or the extravillous trophoblast (EVTs). In humans, the extent of invasion by the extravillous trophoblast can be far extending into the myometrium, unlike that in mouse.

Understanding the molecular mechanisms of mouse placentation is insightful and paves the way to understand events critical for human placentation. However, human and mouse placentation differ significantly with few similarities. Both human and mouse placenta display hemochorial placentation, where the maternal blood bathes in the chorion directly. TGCs in mouse can be

compared to extravillous trophoblast (emanating from the distal cell column cytotrophoblasts) both in terms of gene expression profile and in developmental dynamics only in the first half of gestation. There are distinct species-specific transcriptional regulations underlying the placentation development (Soncin et al., 2018). The T-box DNA binding proteins, Eomesodermin, is not detected in the different gestation weeks of human placenta development, both at the protein and mRNA levels. Contrastingly, Eomes is one of the earlier markers to be associated with TE fate specification in mouse. There also exist similar factors associated with human cytotrophoblast such as VGLL1, not detected in different gestational weeks of mouse development, both at the mRNA and protein level (Soncin et al., 2018). Tead4, the TEA-domain transcription factor associated with CDX2 regulation in TE fate establishment in mouse, on the other hand, display different expression pattern as gestation progresses in both mouse and human. Trophoblast stem cells isolated from mouse and human also differs in terms of the expression of different transcriptional factors instigating differentiation program in these cell types.

Signaling pathways essential for trophoblast development:

Hippo signaling pathway has been attributed as one of the earliest regulators of trophoblast development. Hippo pathway components Tead4 and co-factors Yap/Taz are essential to establish trophectoderm fate by directly regulating Cdx2 expression. In a post-fertilization blastocyst, the outer cells acquire apical protein domains comprising of Par3-Par6-atypical PKCs complex, which sequesters Hippo pathway protein components. Agiomotin (Amot), which remains inactive in outer cells because of its association with apical polarity protein complex, however is localized to the adherens junction in inner apolar cells and mediates Hippo signaling (Hirate et al., 2013). Similarly, Lats2 is apically localized in outside cells but is distributed throughout the cytoplasm in

inner cells where it phosphorylates Amot at Serine 176. This phosphorylation event is essential for Amot activity and also for Amot localization at the adherens junction instead of apical domain. This event stabilizes the interaction of Amot, Nf2 and Lats2, which in turn phosphorylates Yap/Taz leading to sequestration within the cytoplasm and exclusion from the nucleus, thus inhibiting interaction with Tead4 and establishment of TE fate (Cockburn, Biechele, Garner, & Rossant, 2013). Also altered localization of Tead4 in the inner cell mass dictates first mammalian cell fate specification. Lack of nuclear localization of Tead4 in the inner cells impairs TE fate establishment whereas restoration of nuclear Tead4 enables TE transcriptional program in the inner cells (Pratik Home et al., 2012).

Peroxisome proliferator-activated receptor gamma (PPARγ), belongs to the ligand-activated nuclear hormone receptors and has been associated with trophoblast development (Barak et al., 1999; M. M. Parast et al., 2009). PPARγ null mouse are embryonic lethal by E10 and tetraploid chimeras obtained from tetraploid reconstitution were viable suggesting that PPARγ null embryos die of placental defects (Barak et al., 1999). Mouse TS cells, derived from PPARγ null blastocyst, fail to differentiate towards Gcm1 and SynA expressing labyrinth cells (M. M. Parast et al., 2009). PPARγ null mouse TS cells differentiate preferably towards invasive TGCs including canal-associated and parietal TGCs (M. M. Parast et al., 2009). Treatment of primary CTBs with PPARγ agonists, rosiglitazone allowed differentiation of these cells towards SynT lineage indicated by higher hCG secretion. PPARγ overexpression in null mouse TS cells revert back their ability to differentiate towards SynT lineage (M. M. Parast et al., 2009). Hence, the functional importance of PPARγ in terms of both mouse and human SynT development have been well addressed. PPARγ also acts as a transcription factor, binding to specific DNA sequences known as PPARγ-responsive elements (PPREs). Putative PPRE site has been identified on the proximal promoter region of

Gcm1 (Basyuk et al., 1999a; Nakachi et al., 2008; M. M. Parast et al., 2009) thus providing evidence for direct transcriptional regulation. Other gene targets of PPARγ includes Mucin1 (Muc1) which is another important regulator of placental labyrinth development.

GATA family of zinc finger transcription factors have been implicated in the establishment and regulation of trophoblast lineage. GATA factors bind to conserved W(A/T) GATAR(A/G) (GATA motifs) in the upstream promoter sequence of many developmentally important genes. GATA factors, Gata2 and Gata3 were first shown to be important in the regulation of placental lactogen I (*Prl3d1*) and proliferin (*Plf*) in TGCs (Ma et al., 1997; Ng, George, Engel, & Linzer, 1994). Earlier publications from our lab also have shown the importance of Gata3 in pre-implantation mouse embryonic development (Pratik Home et al., 2009). However, Gata2 and Gata3 are individually dispensable for mouse development, however, embryonic lethality results when both proteins are depleted (Pratik Home et al., 2017). Gata2 and Gata3 both regulate a large group of common genes essential to orchestrate stem versus differentiation transcriptional program. The importance of Gata2 and Gata3 in trophoblast development has also been observed in human TE development. GATA2 and GATA3 are highly expressed in vCTBs, cCTBs, nascent EVTs derived from cCTBs (Pratik Home et al., 2017; B. Lee et al., 2016) and CTB derived human trophoblast stem cells (Okae et al., 2018). GATA2 and GATA3 also regulate a number of genes associated with human trophoblast development such as human chorionic gonadotropin alpha subunit (CGA), syncytin, human leukocyte antigen G (HLA-G) to name a few (Cheng & Handwerger, 2005; Ferreira et al., 2016; Steger, Hecht, & Mellon, 1994). GATA2 and GATA3 has been referred to as 'pioneer factors' as these are constitutively expressed in all trophoblast cell types during development, are conserved in trophoblast cells across mammals and directly regulate both activation and repression of trophoblast associated genes (Paul, Home, Bhattacharya, & Ray, 2017).

Cited2, another important regulator for placental development. CITED2 deficiency leads to embryonic lethality at around gestation day 14.5 in mouse (Withington et al., 2006) whereas in rats it is not responsible for embryonic lethality, although it affects placental growth and development. CITED2 deletion causes reduction in the number of trophoblast giant cells, spongiotrophoblast and glycogen cells. There is also defect in fetal vascularization in the placenta (Withington et al., 2006). Cited2 regulates the interaction of transcription factors with CPB/p300 with the transcriptional machinery.

Sirtuin1 (Sirt1), an NAD+ dependent deacetylase, is also one of the important proteins associated with trophoblast lineage development. Earlier studies have shown that absence of Sirt1 causes fetal growth restriction in mouse, but the placenta was not analyzed. However, recent evidences suggest Sirt1 is essential for mouse placenta development, causing embryonic lethality at E13.5, resulting from fetal growth restriction due to blunted trophoblast differentiation (Arul Nambi Rajan et al., 2018). Sirt1 null mouse TSCs display both abrogation of proliferation and differentiation. Sirt1 null mouse TSCs are arrested at the progenitor state where Epcam is also highly co-expressed, indicating the role of Sirt1 in a progenitor population in mouse labyrinth development (Arul Nambi Rajan et al., 2018). Sirt1 deacetylates and leads to the repression of PPARγ. Sirt expression is ubiquitous in both mouse and human placenta.

Hypoxia as a regulator of placentation:

Low oxygen tension has been one of the widely studied factors essential for initial placentation signaling. Hypoxia or low oxygen levels regulates the initial pathways which builds up the vital structures of the placenta. Hypoxia inducible factors or HIFs have 2 subunits –HIFα and HIFβ/ARNT (aryl hydrocarbon receptor nuclear translocator). Upon exposure to high oxygen concentration, HIFs are degraded by prolyl hydroxylase which adds hydroxyl group to the proline

residues and marks them for ubiquitination by von Hippel-Lindau (pVHL) tumor suppressor protein. There are different isoforms of HIFα- HIF1α, HIF2α and HIF3α. HIF1α is ubiquitously expressed, it is present in both CTB and EVT of human first trimester placenta but gradually its expression is diminished at the onset of second trimester with the inflow of blood by dissolution of trophoblast plug and hence establishing normoxic condition. HIF2α and HIF3α are more restricted in their tissue specific expression, whereas HIF2α has been detected both at the RNA and protein level in the placenta. HIF3α is yet to be analyzed in more specific tissues. HIFα dimerizes with ARNT and together they regulate gene expression of hypoxia related signaling. Studies from transgenic mouse models and knockdown analyses of both Hif1α and Hif2α indicates that the HIF components are essential for proper vascularization of the placenta. *Arnt*$^{-/-}$ mouse embryos exhibit midgestation lethality at E9.5-10 and the placentae show reduced and abrogated placental labyrinth and spongiotrophoblast with increased number of trophoblast giant cells (Adelman, Gertsenstein, Nagy, Simon, & Maltepe, 2000; Kozak, Abbott, & Hankinson, 1997). Hif1α deficiency also results in embryonic lethality at E10.5 with abrogated labyrinth development. Additionally, conditional deletion of Hif1α at E8.5 in maternal tissue also results in smaller placentae and disrupted trophoblast invasion. HIF factors are also essential for trophoblast cell fate determination (Cowden Dahl et al., 2005). Hypoxia has been associated with placental pathologies like preeclampsia, placental insufficiency and fetal growth retardation. In preeclampsia, shallow remodeling of spiral arteries has been associated with prolonged hypoxic condition and continued expression of HIF1α well into late gestation. This eventually leads to anti-angiogenic imbalance. However chronic hypoxia, occurring in high altitude pregnancies, needs to be discrete from acute hypoxia. In such cases, IUGR and PE incidences are high but chorangiosis,

increase in fetal capillary density and hence blood supply, acts as the adaptive response ("The Placenta at High Altitude," 2003).

In studies, utilizing both CTB and human pluripotent stem cells, hypoxia condition plays a major role in determining differentiation potential of these cells. Culturing under hypoxic condition, CTBs were shown to lose their 'stemness' potential and differentiate towards EVT population. Recent studies show that culturing first-trimester CTBs in 2% oxygen leads to expansion of HLAG+ EVT population and inhibition of multinucleated syncytium (Wakeland et al., 2017). Another study established that NOTCH+ EVT progenitor population expanded upon culturing first trimester CTBs under hypoxic condition. NOTCH+ progenitor population also showed inhibition of p63, TEAD4 and repressed syncytialization (Haider et al., 2016).

Signaling during human placentation:

The signaling pathways and the different trophoblast progenitor populations in human placenta development still remains obscure due to the ethical limitations and restrictions imposed in studying human embryos and subjects. However recent findings have shed light on the different trophoblast progenitor populations. The trophoblast cells which retain the capacity to proliferate are termed as 'trophoblast progenitors' (TPs), however this proliferative niche of cells becomes more restricted as gestation weeks progresses. Integrin ITGA2 expressing trophoblast progenitor population was identified recently, which was localized towards the base of the cell column cytotrophoblast (cCTB). The ITGA2+ TPs shows intermediate characteristics of both villous cytotrophoblast (vCTB) and extravillous trophoblast (EVT) when compared by gene expression analysis (C. Q. E. Lee et al., 2018). This bipotential nature of the ITGA2+ TPs indicate that they can give rise to both EVTs and VCTs. However mice lacking Itga2 survives with no placental

defects observed (J. Chen, Diacovo, Grenache, Santoro, & Zutter, 2002), hence the functional importance of ITGA2 is more predominant in human placentation. Most of the signaling pathways are highly conserved between mouse and humans, however some of the very important and established signaling pathways for human placentation are discussed below.

Notch signaling in human placentation: Notch signaling is one of the most conserved mechanism in mammals and is important throughout pregnancy (Zhao & Lin, 2012). Notch ligands have 4 receptors (Notch1-4) and ligands Jagged1 (Jag1), Jag2, delta-like proteins (Dll1, Dll3 and Dll4) in mammals. Notch1 shows upregulation due to estrogen and progesterone surge followed by downregulation during preimplantation stages. Notch members are expressed in the developing placenta in the different trophoblast subtypes. Notch expression has also been detected in the progenitor cytotrophoblast population. Notch signaling has been implicated in the differentiation of trophoblast towards EVT (De Falco et al., 2007). Notch signaling has been involved in vasculogenesis and angiogenesis (Kume, 2009). Notch ligands and receptors show expression in vessels of the placental villi. Notch1 receptor and ligands Jag1, Dll1, Dll4 were found to be expressed in the endothelial cells of tertiary villi and perivascular cells. Dll4 was also detected in the capillaries of the placental villi (Herr, Schreiner, Baal, Pfarrer, & Zygmunt, 2011).

HCG signaling in human placentation: The human chorionic gonadotropin (hCG) hormone is essential throughout pregnancy, including decidualization, implantation, differentiation and trophoblast cell fusion. hCG is required for fusion of villous cytotrophoblasts to form syncytiotrophoblast (Shi, Lei, Rao, & Lin, 1993). hCG belongs to a family of glycoproteins having a common α subunit and unique β subunit. hCG ligands upon binding to the hCG receptors, which are G-protein coupled receptor, having 7 transmembrane domains, activates downstream adenylyl cyclase, phospholipase C and ion channels further releasing intracellular messengers like cAMP,

Ca^{2+} and inositol phosphate. During trophoblast fusion, there is downregulation in the CG receptor and transcripts while hCG ligand expression increases and is generated in huge amounts. This has been tested by in vitro differentiation of cytotrophoblasts (Pidoux et al., 2007). During fusion process, hCG has a biphasic effect on cadherin expression which is a cell adhesion molecule and allows cellular aggregation (Shi et al., 1993).

Protein kinase C isoforms:

The protein kinase C isoforms are a group of serine-threonine kinases which have been implicated in various functions within the cellular system. Earlier reports suggest that PKC signaling is very critical in cellular proliferation, differentiation, cell cycle control, cell survival and polarity. Deregulation of any signaling molecule in this vital signal transduction circuitry can lead to manifestation of several diseases like cancer. There are around 11 isoforms of PKC known in mammals and these have been classified based on the distribution of activation domains and functions. The PKC isoenzymes are divided into three main subtypes- classical PKCs (α, βI, βII, γ), novel PKCs (δ, ε, η, θ, μ) and atypical PKCs (λ/ι, ζ). Each of these subgroups has an N terminal isoenzyme-specific regulatory domain, a C terminal kinase domain and a variable hinge region (Figure 4). The isoenzymes differ in the amino acid sequence and also presence or absence of protein domains. **Classical or conventional PKCs (α, βI, βII, γ) -** These have functional C1A and

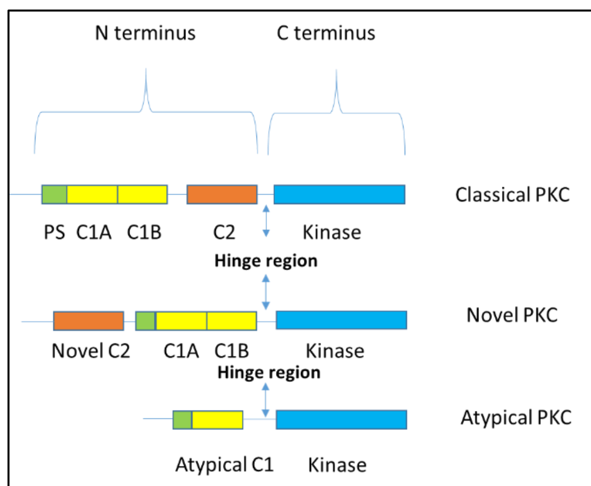

Figure 4: The structure of the domains of the PKC isoforms. The atypical C1 domain is totally different from the other PKC isoform domains. PS- pseudosubstrate binding site. Adapted with permission from (Newton, 2001). Copyright (2001) American Chemical Society.

C1B domains, which are cysteine-rich motifs, bind phosphatidylserine and diacylglycerol (DAG)/phorbol esters and a C2 domain which binds anionic lipids and Ca^{2+}. The members of this subfamily require Ca^{2+} and diacylglycerol for induction of their catalytic activity. **Novel PKCs (δ, ε, η, θ, μ)** - These lack the C2 domain and thus are Ca^{2+} independent but require DAG or phorbol ester for activation. The novel group members play a significant role in maintaining stem cell self-renewal. **Atypical PKC (ζ and λ/ι) -** These have unique N terminal structure with a single cysteine-rich motif, not repeated in tandem like the other traditional PKC isoforms and lack binding site for Ca^{2+}, that is why they are named as 'atypical'. These neither require DAG nor Ca^{2+} for their activation, instead they are activated by other phospholipids like PS, phosphatidic acid or inositol lipids. It is still not fully established how this group of kinases gets activated. In this study, our focus will be on the atypical group of Protein Kinase C isoforms.

Sequence conservation between PKC λ/ι and other PKCs:

The gene coding PKC λ/ι, *Prkci,* was cloned almost 20 years back based on the amino acid sequence similarity to PKC ζ (Ono et al., 1989; Selbie, Schmitz-Peiffer, Sheng, & Biden, 1993). The human PKC ι and the mouse PKC λ are orthologs with 98% sequence similarity hence it is referred to as PKC λ/ι (Akimoto et al., 1994). PKC λ/ι shows 86 % sequence similarity with PKC ζ and 45-55% with other PKCs. The cysteine rich motif also shows 69% amino acid homology with PKC ζ and only 30-40% with other PKCs (Akimoto et al., 1994). The sequence similarity between PKC λ/ι and PKC ζ hampers isotype-specific effect of dominant negative mutants. In addition to that, PKC ζ antibodies cross react with PKC λ/ι and this makes conclusions inferred from experiments complex.

Activation of aPKCs:

The enzymological functions and mode of activation of the aPKCs differ from the other PKCs due to the structural modifications and the unique atypical N–terminus. Most of the literature studies have been conducted for PKC ζ, so here we will assume that their activation pathway is mostly similar. The insensitivity of PKC ζ to DAG or phorbol esters was shown in a previous study on cloning (Akimoto et al., 1994). PKC λ/ι is activated downstream of phosphoinositide 3-kinase (PI3K) in intact cells (Akimoto et al., 1996). So PKC λ/ι plays a critical role in relaying signals to particular targets downstream of PI3K (Herrera-Velit, Knutson, & Reiner, 1997; Kotani et al., 1998; Liu, Ning, Dantzer, Freund, & Kelley, 1998; Mendez, Kollmorgen, White, & Rhoads, 1997; Standaert et al., 1997). Literature mining suggest that phosphatidylinositol-3,4,5-$(PO_4)_3$ (PIP3) also interacts with cysteine-rich sequences, uplifts the pseudo-substrate inhibition by inducing conformational changes thereby activating aPKCs. Previous studies have reported that phosphoinositide-dependent kinase 1(PDK1) also activates PKC ζ in vitro in a PIP3-dependent manner by phosphorylating Thr(T410) which is located in the activation loop of PKC ζ (Chou et al., 1998; Dong et al., 1999; Le Good et al., 1998) . Amino acid substitution of Thr to Ala further showed that it abrogates the kinase activity (Perander, Bjorkoy, & Johansen, 2001). Direct interaction of PIP3 with the cysteine–rich region of aPKCs leads to their acute activation (Standaert, Bandyopadhyay, Kanoh, Sajan, & Farese, 2001). PDK-1 mediated activation in aPKCs differs from that in traditional PKCs. Phosphorylation induces chronical maturation of the traditional PKCs which then undergoes acute activation by DAG (Newton, 2001).

Interaction between aPKCs and other proteins:

Earlier studies show that PKC λ/ι and PKC ζ play similar roles in terms of kinase activity and interaction with other protein complexes (apart from LIP, which binds specifically to PKC λ/ι). The high degree of sequence similarity and the absence of specific antibodies make dissecting out functional difference between the two members even more difficult. So far, pseudo-substrate peptides, antisense oligonucleotides and dominant negative mutants and knockout mouse models have been used to show the phenotype when PKC λ/ι is inhibited.

PB1 domain binding proteins- The atypical N-terminal region of aPKCs has a unique sequence, the V1 region, with a short acidic stretch named as OPR/PC/AID (Moscat & Diaz-Meco, 2000; Ponting et al., 2002). This motif is also present in yeast cell polarity protein Cdc24p and mammalian phagocyte-specific factor $p40^{phox}$ and is required for binding of polarity determinant scaffold protein (Bem1p) and one of the cytosolic subunit of NADPH oxidase ($p67^{phox}$) (Diaz-Meco & Moscat, 2001). The V1 region of PKC λ/ι also contains a structural module PB1 domain (a.a 19-108) which was first identified in Bem1p and $p67^{phox}$ in their binding site for Cdc24p and $p40^{phox}$ respectively (Nakamura et al., 1998). P62/ZIP and PAR-6 cell polarity proteins also binds to the V1 region of aPKCs (Moscat & Diaz-Meco, 2000; Suzuki et al., 2001). Hence the PB1 domain plays a critical role in allocating aPKCs to different signaling pathways (Diaz-Meco & Moscat, 2001).

PAR-6 links aPKCs to small GTPases of the Rho family, Rac1 and Cdc42 by its CRIB/PDZ domain (Joberty, Petersen, Gao, & Macara, 2000; Lin et al., 2000; Noda et al., 2001; Suzuki et al., 2001). This interaction is GTP dependent. It has been observed that the kinase activity of aPKCs is more pronounced when recombinant Cdc42 preloaded with GTP-γS but not with GDP (Yamanaka et al., 2001). PAR-6 mutants lacking the CRIB/PDZ domain interestingly showed

kinase activity independent of Cdc42. It is hypothesized that PAR-6 binding to aPKCs suppresses its kinase activity which is released upon Rac1/Cdc42 binding but there is lack of experimental evidence to confirm this.

Cysteine-rich domain binding protein- Prostate androgen response-4 (Par-4), which is different from polarity protein PAR-4, has been shown to bind to the cysteine-rich domain of aPKCs. This protein is expressed upon induction of apoptosis. Diaz—Meco *et al.* have reported that it interacts with a single-cysteine rich sequence of both PKC λ/ι and PKC ζ, suppressing their kinase activity and hence inducing apoptotic pathway (Diaz-Meco, Lallena, Monjas, Frutos, & Moscat, 1999). On the other hand, Lamda –interacting protein (LIP) also has been shown to interact with the cysteine-rich domain of PKC λ/ι specifically but this protein acts as an activator (Diaz-Meco, Municio, Sanchez, Lozano, & Moscat, 1996).

PXXP motif-binding protein- Wooten *et al.* has shown that human PKC λ/ι interacts with the SH3 domain containing protein src (Wooten, Vandenplas, Seibenhener, Geetha, & Diaz-Meco, 2001). Amino acid sequence 96-111 rich in PXXP motif is responsible for this interaction. This association is enhanced in the presence of NGF and leads to the formation of a complex involving NGF receptor (TrkA), src and PKC λ/ι. The PXXP motif is only present in the aPKCs, hence this interaction is also restricted to only the aPKCs. *In vitro* purified PKC λ/ι gets phosphorylated at specific Tyrosine residues in the kinase domain by src. This finding shows a novel mode of activation of PKC λ/ι through the PXXP motif.

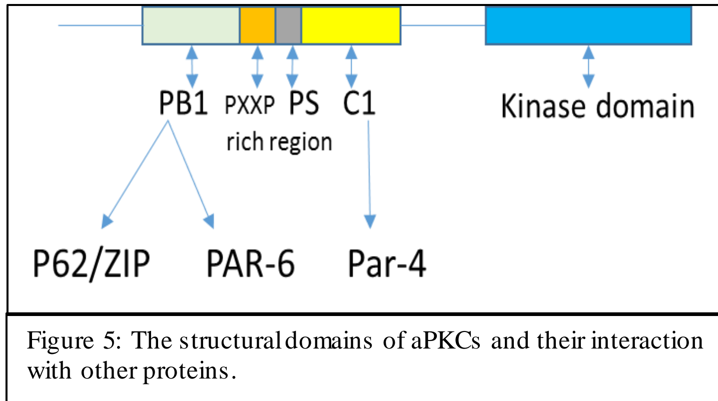

Figure 5: The structural domains of aPKCs and their interaction with other proteins.

Functions of atypical PKC λ/ι and PKC ζ:

Atypical PKCs are one of important component of cell polarity assigning PAR complex. Par3, Par6 and aPKCs together constitutes the cell polarity complex which attributes to the polarized organization of proteins and organelles within the eukaryotic cells. One of the important functions of the cell polarity complex is in the trabeculation of myocardium during embryonic heart development. *Prkci* null mouse mutants display improper localization of the cell polarity complex on the luminal side of the myocardial cells leading to abnormal perpendicular cell division effecting myocardial trabeculation in the embryonic heart (Passer, van de Vrugt, Atmanli, & Domian, 2016). *Prkci* null mouse heart also show trabeculation defect (Passer et al., 2016).

PKC λ/ι, as a component of the cell polarity complex, has also been associated with cell cavitation. Cavitation is essential for the generation of a hollow structure from a solid block of cells during cellular morphogenesis. Cavitation occurs during embryogenesis. Proper localization of cell polarity complex proteins is essential for undergoing epithelial morphogenesis. *Prkci* null embryoid bodies (EBs) show discontinuous adherens junction and improper tight junction formation. *Prkci* null EBs also display aberrant localization of apical markers like GM130, Podocalyxin (I. K. Mah et al., 2016), Ezrin (Wald et al., 2008), Mupp1 and Par3 (Seidl et al., 2013). Hence, PKC λ/ι is essential for the proper recruitment of these polarity proteins. *Prkci* null EBs display improper cavitation, not due to reduced apoptosis of cells as *Prkci* null EBs show similar apoptotic index when compared to control EBs. There is neither major difference in the

proliferation rate in *Prkci* null EBs and control EBs. The inability of *Prkci* null EBs to cavitate was partially rescued by Ezrin overexpression (I. K. Mah et al., 2016) which is considered to be one of the downstream regulators of PKC λ/ι signaling. However, wildtype R1 ES cells when mixed with null EBs fully rescued ability to cavitate (I. K. Mah et al., 2016), thus establishing the role of a polarity-competent cells to exert a non-cell-autonomous influence to recover cavitation.

Since atypical PKCiota and PKCzeta are components of the cell polarity complex, studies trying to understand the functional importance of the complex in determining polarization of different cell types and tissue homeostasis have been reported. Constitutive hematopoietic-specific deletion of PKCiota and PKCzeta have shown that there is no effect on the polarization, self-renewal and repopulation activity of hematopoietic system both in fetal and adult mouse (Sengupta et al., 2011). Serial competitive repopulation assay also revealed that neither PKCiota nor PKCzeta have any functional role in regulating steady-state hematopoiesis (Sengupta et al., 2011).

Atypical PKCiota as a component of cell polarity complex: The most important function of PKC λ/ι is it being the component of the trimeric protein complex PAR3-PAR6-aPKC which regulates cell polarity in *C. elegans*, *Drosophila*, *Xenopus,* zebrafish and mammalian cells (Tabuse et al., 1998)**.** PKCs along with PAR proteins are essential for the regulation of assymetric divisions in the blastomere stages of *Caenorhabditis elegans*. Infact, atypical PKCs were identified from a screen for proteins that bind PAR-1 and is essential for successful asymmetric divisions and proper distributions of PAR proteins. PAR proteins PAR-1 and PAR-2 show reciprocal localization compared to PAR-3 in blastomeres of C.elegans. PAR-3 is considered to be the most important component assigning cell polarity cues to the cells undergoing asymmetric division. In atypical PKC mutants, known as pkc-3 in C. elegans, the distribution of the PAR proteins, especially PAR-3 becomes abrogated. *Pkc-3* mutants display embryonic arrest and lethality, resembling PAR-3

like phenotypes (Tabuse et al., 1998). Thus, pkc-3 plays an indispensable role in *C. elegans* embryonic polarity establishment along with PAR-3 and other PAR proteins.

Role in signaling pathways: PKC λ/ι relays growth factor mediated signals from PI3K and/or Ras to the activation of the MAPK cascade (Berra et al., 1995; Bjorkoy, Perander, Overvatn, & Johansen, 1997)**.** Several isoforms including PKC ζ plays a critical role in the activation of cyclin D1 by oncogenic Ras. This process is MEK-1, Rac-1 dependent and PKC ζ is required downstream of MEK-1 to activate ERK signaling (Kampfer et al., 2001). PKC ζ is also involved in the regulation of Iκκβ and not of Iκκα in response to TNFα (Lallena, Diaz-Meco, Bren, Payá, & Moscat, 1999). *Lallena et al* also showed that aPKCs interact and phosphorylates Iκκβ both *in vitro* and *in vivo*. Transfection of PKC ζ dominant negative mutant severely impairs Iκκβ but not Iκκα in TNF-α stimulated cells, whereas recombinant active PKC ζ stimulates *in vitro* Iκκβ activity but does not have any effect on Iκκα in unstimulated cells. Thus, aPKCs play an important role in NF-κβ pathway. aPKCs also interact with RIP, a death domain kinase, via p62 and regulates NF-κβ activation through TNF-α signaling cascade. RIP associates with TNF receptor1 (TNF-R1) through the adapter molecule, TRADD (Sanz, Sanchez, Lallena, Diaz-Meco, & Moscat, 1999)**.** p62 links aPKCs to RIP and aPKCs link Iκκβ to the RIP/TRADD/TNF-R1 complex. Expression of a dominant negative mutant of PKC λ/ι abrogates RIP-stimulated NF-κβ activation (Hsu, Huang, Shu, Baichwal, & Goeddel, 1996)**.**

Role in cancer progression: PKC λ/ι is a bonafide human oncogene. PKC iota gene has been associated with tumor-specific gene amplification in multiple types of cancer. It is required for the transformed growth of cancer cells. PKC λ/ι is a relevant downstream target of Bcr/Abl mediated apoptotic resistance to K562 chronic myelogenous leukemia cells (Jamieson, Carpenter, Biden, & Fields, 1999)**.** In another report, Jiang *et al* showed that dietary fats like fish oil and pectin can

modulate PKC λ/ι expression at different stages of colon cancer (Jiang, Lupton, & Chapkin, 1997). Fish oil block carcinogen-induced elevated PKC λ/ι membrane localization whereas pectin increases cytosolic PKC λ/ι levels in comparison to cellulose. PKC λ/ι is also required for Ras-mediated colon carcinogenesis *in vivo* (Murray et al., 2004). PKC λ/ι is also required for transformed growth of non-small cell lung cancers (NSCLC) which does not harbor K-ras mutations, knockdown inhibits tumorigenicity *in vivo* (Regala, Weems, Jamieson, Copland, et al., 2005). PKC λ/ι also functions in NSCLC cell survival, resistance to chemotherapy and invasion *in vitro* (Jin, Xin, & Deng, 2005). PKC λ/ι protein is overexpressed in vast primary NSCLC tumors (Regala, Weems, Jamieson, Khoor, et al., 2005), pancreatic tumors (Evans, Cornford, Dodson, Neoptolemos, & Foster, 2003) and chronic myeloid leukemia (Gustafson et al., 2004). Elevated level of PKC λ/ι is observed in ovarian cancer patients, where its level of expression correlates with stages of tumor indicating that PKC λ/ι contributes to tumor progression and aggressiveness (L. Zhang et al., 2006).

Role of aPKCs in the context of stem cells: Niessen *et al* showed that aPKCs is crucial for the homeostasis of self-renewing stratifying epithelia and regulation of cell fate, differentiation and maintenance of epidermal bulge stem cells. Loss of PKC λ/ι leads to loss of quiescent hair follicle bulge stem cells and increases the proliferating progenitor population. Loss of PKC λ/ι also increases the asymmetric divisions in different compartments including the bulge (Niessen et al., 2013). PKC ζ suppresses intestinal stem cell function by promoting downregulation of β-catenine and YAP through direct phosphorylation (Llado et al., 2015). Chemical inhibition and RNA knockdown approaches have suggested that atypical PKC isoforms are required for asymmetric cell division of CD8+ T cells, to control the balance between self-renewal and differentiation in *Drosophila* and *C. elegans* (Chang et al., 2011; Metz et al., 2015; Oliaro et al., 2010). Atypical

PKCs have also been implicated in CD4+ T cell differentiation (Martin et al., 2005; J.-Q. Yang, Leitges, Duran, Diaz-Meco, & Moscat, 2009).

Atypical PKCs and ES cells:

The atypical PKCs have been shown earlier participating in various signaling cascades. Dutta *et al* reported the role of aPKCs in regulating NF-κβ in the context of embryonic stem cells (Debasree Dutta et al., 2011). For the first time, it was reported that inhibition of aPKCs play a critical role in maintaining ES cells in their self-renewal state which is independent of inhibition of ERK/GSK3 or activation of Stat3 signaling pathways. A highly specific inhibitor, called Gö 6983, which inhibits specifically PKC isoforms α, β, γ, δ, ζ, μ (Gschwendt et al., 1996; Mor-Vaknin, Punturieri, Sitwala, & Markovitz, 2003) was used to inhibit angiogenic signal-mediated gene expression in endothelial cells. Dutta *et al* reported that inhibition of PKC ζ could maintain ES cells in their proliferative self-renewal condition even in the absence of LIF. The E14 ES cells were maintained until 18 consecutive passages and the colonies had identical morphology as well as expression of pluripotency markers like Oct4, Nanog and Sox2. There was no induction of differentiation markers. Dutta *et al* also reported that they obtained the same results when they used R1 ES cells which depicts that inhibition of PKC maintain ES cells in their self-renewal state irrespective of cell types. With the withdrawal of the inhibitor Gö 6983, the mouse ES cells again readily underwent differentiation and formed embryoid bodies when grown on collagen IV. The inhibitor treated ES cells, when injected into blastocyst, generated adult chimeras. These produced germline offsprings when crossed with C57BL/6 adults. So, these results indicate that inhibition of PKC signaling is sufficient to derive germline –competent pluripotent ES cells (Figure 6).

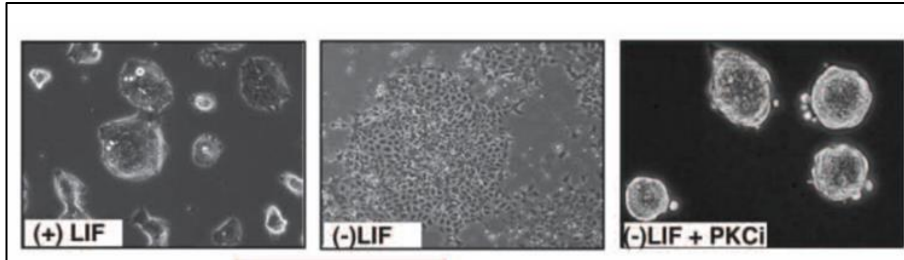

Figure 6: Images of E14 ES cells showing undifferentiated colony morphology in the presence of LIF and in the presence of PKCi (Gö 6983) even when LIF is withdrawn from the culture condition. Adapted from (Debasree Dutta et al., 2011).

Duran *et al* reported that phosphorylation of a key Serine 311 residue on RelA subunit of NF-κβ is important for maintaining stem cell pluripotency (Duran, Diaz-Meco, & Moscat, 2003). Inhibition of PKC ζ by Gö 6983 also inhibits NF-κβ signaling in mouse ES cells**.** Thus NF-κβ is a downstream target of PKC ζ and contributes to the lineage commitment in mouse ES cells.

Rajendran *et al* in 2013 also reported a similar study but in rat system where they showed that inhibition of PKC signaling is sufficient to maintain rat ES cells in the self-renewal pluripotent state (Ganeshkumar Rajendran et al., 2013). Maintenance of repressive marks like trimethylation of H3K27 (H3K27Me3) by polycomb repressive complex2 (PRC2) at the developmental regulator genes, has been implicated in stem cell pluripotency (Boyer et al., 2006; Pasini, Bracken, Hansen, Capillo, & Helin, 2007). Rajendran et al reported that Gö 6983 treatment maintains PRC2 mediated H3K27Me3 mark at the different developmental regulator genes. In contrast H3K27Me3 mark and PRC2 was not detected at the pluripotency genes like Oct4 and Nanog. On the other note, suppression of miR-21, miR-29a and miR-34a has been implicated in maintaining ESC-like phenotype (Choi et al., 2011; Singh, Kagalwala, Parker-Thornburg, Adams, & Majumder, 2008; C.-S. Yang, Li, & Rana, 2011). Rajendran *et al* reported that downregulation of PKCζ is associated with repression of miR-21 and miR-29a in rat ES cells whereas expression of miR-21, miR-29a in PKCζ KD rat ES cells leads to loss of undifferentiated morphology, loss of expression of pluripotency genes and upregulation of differentiation markers. Thus miR-21, miR-29a induction

also contributes to ES cell differentiation and when repressed, due to PKCζ inhibition, helps maintaining ES cells in their undifferentiated stem state.

Mahato *et al* reported in 2014 that PKC λ/ι depletion helps maintain ES cells in their pluripotent state much like that of PKC ζ (Biraj Mahato et al., 2014). This report took into consideration the role of mitochondrial function with respect to stem cell pluripotency. Pluripotent ES cells contain immature mitochondria and rely on glycolysis for energy supply (Folmes et al., 2011; Mandal, Lindgren, Srivastava, Clark, & Banerjee, 2011; Prowse et al., 2012; J. Zhang et al., 2016; Zhou et al., 2012). Inhibition of glycolysis promotes differentiation of ES cells (Ezashi, Das, & Roberts, 2005; Prowse et al., 2012; Varum et al., 2009). Mahato *et al* reported that knocking down PKC λ/ι abrogates mitochondrial biogenesis (by inhibiting mtDNA synthesis) and maturation (mitochondria lack proper cristae formation and appear globular in shape in PKC λ/ι depleted cells) and thus promotes glycolytic pathway for energy metabolism. This in turn helps retaining ES cells in their pluripotent state. There are five membrane bound protein complexes called the electron transport chain which transmits electron and pumps out proton into the inter-membrane space which leads to the generation of an electrochemical gradient, the energy of which is used by the ATP synthase to generate ATP from ADP and Pi. PKC λ/ι depletion inhibits functions of one of these membranes bound protein complexes, NADH dehydrogenase by inhibiting the complex I assembly factors. Hypoxia-inducible factor 1α (HIF1α) has been shown upregulating expression of Oct4 and Nanog (Mathieu et al., 2011). HIF1α also regulates mitochondrial function and promotes metabolic shift towards glycolysis in ES cells (Pereira et al., 2013; Prigione et al., 2014; Zhou et al., 2012). Loss of PKC λ/ι strongly stabilizes HIF1α protein in ES cells. Mahato *et al* reported that downstream of PKC λ/ι depletion, HIF1α plays a major role in maintaining

pluripotency of ES cells by altering mitochondrial functions in ES cells and promoting glycolysis, thereby preventing their differentiation.

A recent study in 2015 by Mah *et al* suggested that PKC iota is required for expansion of stem cell types and it does so by regulating Notch signaling (In Kyoung Mah, Soloff, Hedrick, & Mariani, 2015). They used an in vitro approach in order to bypass the embryonic lethality upon PKC λ/ι depletion and reported that absence of PKC λ/ι leads to the generation of multiple stem cell types including cells with primordial germ cells (PGC) like characteristics. The authors used embryoid bodies which can develop like the early post implantation embryo and can be cultured long enough to observe differentiation patterns. In this report, null EBs show generation of more progenitor populations like neural, cardiac and primordial germ cells. Staining of *Prkci* null EBs for ISL1 (a cardiac stem cell marker), PAX6, NESTIN (neural stem and progenitor markers) also showed more cells staining positive for these markers compared to the heterozygous EBs. Treatment of null and heterozygous EBs with inhibitor Gö 6983 showed more staining for Stella (a PGC marker) in the null EBs. Hence loss of PKC λ/ι leads to the generation of multiple stem cell or progenitor cells. This report also showed that NUMB, a component of the NOTCH pathway gets directly phosphorylated by PKC λ/ι and acts downstream of it. Absence of PKC λ/ι results in loss of phosphorylation at the most well characterized- Ser276 of NUMB (Smith et al., 2007). Notch pathway inhibition by activated NUMB has been reported in mammals and flies in earlier studies (Berdnik, Török, González-Gaitán, & Knoblich, 2002; French et al., 2002). Mah *et al* reported that NUMB inhibition by loss of PKC λ/ι mediated phosphorylation, leads to upregulation of NOTCH1 activity and downstream transcriptional targets. Hence, if summarized, loss of PKC λ/ι results in NUMB inactivation which causes upregulation of NOTCH activity and generation of multiple stem cell and tissue progenitors like neural stem cells, cardiomyocytes and erythrocyte progenitors.

Thus, PKC λ/ι plays a vital role in stem cell fate determination and maintenance of balance between self-renewal and differentiation.

Role of aPKCs in the context of human ES cells: Human ES cells offer insights into the developmental processes as these events cannot be studied directly inside a human embryo and these cells holds the answer for clinical areas like birth defects, pregnancy loss and infertility. Thomson *et al* isolated and cultured human ES cells for the first time from the inner cell mass of in-vitro fertilized embryos, obtained from consented, informed individuals. These human ES cells have normal karyotypes, express high levels of telomerase activity and express cell surface markers of undifferentiated ES cells like SSEA-3,SSEA-4, TRA-1-60 , TRA-1-81, alkaline phosphatase that characterize primate embryonic stem cells (Thomson et al., 1998). The ES cells were passaged for months and cryopreserved. The human ES cells maintained the potential to form all the three germ layers- ectoderm, mesoderm and endoderm. These gave rise to teratomas when injected into SCID-beige mice (Thomson et al., 1998).

Many reports showed that FGF2 activates both the MAPK/ERK and PI3K/AKT pathways which are essential for maintaining pluripotency in human ES cells (Amit et al., 2000; Ding et al., 2011; Laslett, Filipczyk, & Pera, 2003; Xu et al., 2005). FGF2 also induces PKC isoforms δ, ε, ζ which in turn phosphorylates GSK-3β, directly or indirectly, promoting differentiation of human PS cells (Kinehara et al., 2013). On the contrary regulation of GSK-3β by PI3K/AKT signaling supports self-renewal of human ES cells. Thus, PKC isoforms play a vital role in providing differentiation cues to human ES cells.

Apart from these known functional attributes of aPKCs, no further studies have been conducted on deducing the functional importance of aPKCs in the context of embryonic stem or trophoblast

stem cells. The functional role of aPKCs in trophoblast stem cells remains unknown. Hence, we wanted to expand our knowledge and understand the functional importance of aPKCs in trophoblast lineage development which is described in the next two chapters.

Chapter2: Atypical Protein Kinase C iota (PKCλ/ι) Ensures Mammalian Development by Establishing the Maternal-Fetal Exchange Interface

This work was previously published as a preprint article in BioRχiv and reprinted here with additional text. *Bhaswati Bhattacharya, Pratik Home, Avishek Ganguly, Soma Ray, Ananya Ghosh, Rashedul Islam, Valerie French, Courtney Marsh, Sumedha Gunewardena, Hiroaki Okae, Takahiro Arima, Soumen Paul. BioRχiv. doi: https://doi.org/10.1101/843375 (November 2019).*

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INTRODUCTION

Trophoblast progenitors are critical for embryo implantation and early placentation. Defective development and differentiation of trophoblast progenitors during early human pregnancy either leads to pregnancy failure (K. Cockburn & J. Rossant, 2010; Pfeffer & Pearton, 2012; R. M. Roberts & S. J. Fisher, 2011; J. Rossant & J. C. Cross, 2001), or pregnancy-associated complications like fetal growth restriction and preeclampsia (Myatt, 2006; Pfeffer & Pearton, 2012; Redman & Sargent, 2005; J. Rossant & J. C. Cross, 2001), or serves as the developmental cause for postnatal or adult diseases (Funai et al., 2005; Gluckman, Hanson, Cooper, & Thornburg, 2008; Godfrey & Barker, 2000). However, due to experimental and ethical barrier, we have a poor understanding of molecular mechanisms that are associated with early stages of human placentation. Rather, gene knockout studies in mice have provided important information about molecular mechanisms that regulate mammalian placentation. While mouse and human placentae differ in their morphology and trophoblast cell types, important similarities exist in the formation of the maternal-fetal exchange interface. Both mice and humans display hemochorial placentation (Carter, 2007), where the maternal-fetal exchange interface is established via direct contact between maternal blood and placental SynTs.

In a peri-implantation mouse embryo, proliferation and differentiation of polar trophectoderm results in the formation of TSPCs, which reside within the extra-embryonic ectoderm ExE (Rossant, 2001), and later in ExE-derived ectoplacental cone (EPC) and chorion. Subsequently, the TSPCs within the ExE/EPC region contribute to develop the junctional zone, a compact layer of cells sandwiched between the labyrinth and the outer TGC layer. Development of the junctional zone is associated with differentiation of trophoblast progenitors to four trophoblast cell lineages (i) TGCs (Simmons, Fortier, & Cross, 2007), (ii) spongiotrophoblast cells (Simmons & Cross, 2005), (iii) glycogen cells

and (iv) invasive trophoblast cells that invade the uterine wall and maternal vessels (Kaufmann, Black, & Huppertz, 2003; Rosario, Konno, & Soares, 2008; Soares et al., 2012).

The mouse placental labyrinth, which constitutes the maternal-fetal exchange interface, develops after the allantois attaches with the chorion. The multilayered chorion forms around embryonic day (E) 8.0 when chorionic ectoderm fuses to basal EPC, thereby reuniting TSPC populations separated by formation of the ectoplacental cavity (D. G. Simmons et al., 2008). Subsequently, the chorion attaches with the allantois to initiate the development of the placental labyrinth, which contains two layers of SynTs, known as SynT-I and SynT-II. At the onset of labyrinth formation, Glial Cells Missing 1 (*Gcm1*) expression is induced in the TSPCs of the chorionic ectoderm (Basyuk et al., 1999a), which promotes cell cycle exit and differentiation to the SynT-II lineage (D. G. Simmons et al., 2008). Whereas the TSPCs of the basal EPC progenitors that express Distal-less 3 (*Dlx3*), contributes to syncytial SynT-I lineage (D. G. Simmons et al., 2008).

In contrast to mice, the earliest stage of human placentation is associated with the formation of a zone of invasive primitive syncytium at the blastocyst implantation site (Boss, Chamley, & James, 2018; James, Carter, & Chamley, 2012; Knofler et al., 2019). Later, columns of CTB progenitors penetrate the primitive syncytium to form primary villi. With the progression of pregnancy, primary villi eventually branch and mature to form the villous placenta, containing two types of matured villi; (i) anchoring villi, which anchor to maternal tissue and (ii) floating villi, which float in the maternal blood of the intervillous space (Boss et al., 2018; James et al., 2012; Knofler et al., 2019). The proliferating CTBs within anchoring and floating villi adapt distinct differentiation fates during placentation (Haider et al., 2016). In anchoring villi, CTBs establish a column of proliferating CTB progenitors known as column CTBs (Haider et al., 2016), which differentiate to invasive extravillous trophoblasts (EVTs), whereas, CTB progenitors of floating villi (villous CTBs) differentiate and fuse

to form the outer multinucleated SynT layer. The villous CTB-derived SynTs establish the nutrient, gas and waste exchange surface, produce hormones and promote immune tolerance to fetus throughout gestation (Beer & Sio, 1982; Chamley et al., 2014; Cole, 2012; Costa, 2016; PrabhuDas et al., 2015; M. Yang, Lei, & Rao Ch, 2003).

Thus, the establishment of the placental exchange surface in both mice and humans are associated with the formation of differentiated, multinucleated SynTs from the trophoblast progenitors of placenta primordia. Moreover, both mouse and human trophoblast progenitors express key transcription factors like, GCM1, DLX3, Peroxisome proliferator-activated nuclear receptor gamma (PPARγ) and GATA binding protein 2 (GATA2), which have been shown to be important for SynT development during placentation (Anson-Cartwright et al., 2000b; K. Cockburn & J. Rossant, 2010; P. Home et al., 2017; Mana M. Parast et al., 2009; Pfeffer & Pearton, 2012; R. M. Roberts & S. J. Fisher, 2011; Rossant, 2004; Janet Rossant & James C. Cross, 2001; Stecca et al., 2002). Despite these similarities, conserved signaling pathways that program SynT development in both mouse and human trophoblast progenitors are incompletely understood. Fortunately, the success in deriving true human TSCs from villous cytotrophoblast cells (CTBs) (Okae et al., 2018) have opened up new possibilities for direct assessment of conserved mechanisms that prime differentiation of multipotent trophoblast progenitor to SynT lineage. Therefore, we herein analyzed both mouse mutants and human TSCs to test the specific role of PKCλ/ι in that process.

The PKCλ/ι belongs to the atypical group of PKCs, which consists of another isoform PKCζ. The aPKC isoforms have been implicated in cell lineage patterning in preimplantation embryos (Zhu, Leung, Shahbazi, & Zernicka-Goetz, 2017). We demonstrated that both PKCζ and PKCλ/ι regulate self-renewal vs. differentiation potential in both mouse and rat embryonic stem

cells (D. Dutta et al., 2011; B. Mahato et al., 2014; G. Rajendran et al., 2013). Interestingly, gene knockout studies in mice indicated that PKCζ is dispensable for embryonic development (Leitges et al., 2001), whereas ablation of PKCλ/ι results in early gestation abnormalities leading to embryonic lethality (Seidl et al., 2013; Soloff, Katayama, Lin, Feramisco, & Hedrick, 2004) prior to embryonic day (E) 9.5, a developmental stage equivalent to first trimester in humans. However, importance of PKCλ/ι in the context of post-implantation trophoblast lineage development during mouse or human placentation has never been addressed. We found that PKCλ/ι protein is specifically abundant in TSPCs and villous CTBs within the developing mouse and human placenta, respectively. We show that both global and trophoblast-specific loss of PKCλ/ι in a mouse embryo is associated with defective development of placental labyrinth due to impairment of gene expression programming that ensures SynT development. We further demonstrate that the PKCλ/ι-signaling in human TSCs is also essential for maintaining their SynT differentiation potential. Our analyses revealed an evolutionarily conserved, developmental stage-specific mechanism in which PKCλ/ι-signaling orchestrates gene expression program in trophoblast progenitors for successful progression of in utero mammalian development.

MATERIALS AND METHODS

Ethics Statement regarding studies with mouse model and human placental tissues: All studies with mouse models were approved by IACUC at the University of Kansas Medical Center (KUMC). Human placental tissues (6th-9th weeks of gestation) were obtained from legal pregnancy terminations via the service of Research Centre for Women's and Infants' Health (RCWIH) BioBank at Mount Sinai Hospital, Toronto, Canada. The Institutional Review Boards at the KUMC and at the Mount Sinai hospital approved utilization of human placental tissues and all experimental procedures.

Collection and analyses of mouse embryos: Preimplantation embryos were isolated at E2.5 and cultured in KSOM to form matured blastocyst. 10 embryos were used to test for PKCλ/ι expression via immunostaining. Expressions analyses of PKCλ/ι in postimplantation mouse embryos (from CD1 and Sv/129 strains) were performed at developmental stages such as E7.5, 9.5 and 12.5. At least 10 embryos at each developmental stage were used for the PKCλ/ι expression studies. The *Prkci* knockout mice (B6.129-*Prkci*^{tm1Hed}/Mmnc) were obtained from the Mutant Mouse Regional Resource Center, University of North Carolina (MMRRC UNC). The heterozygous animals were bred to obtain litters and harvest embryos at different gestational days. Pregnant female animals were identified by presence of vaginal plug (gestational day 0.5) and embryos were harvested at various gestational days. Uterine horns from pregnant females were dissected out and individual embryos were analyzed under microscope. Tissues for histological analysis were kept in dry-ice cooled heptane and stored at -80 for cryo-sectioning. Yolk sacs from each of the dissected embryos were collected and genomic DNA preparation was done using Extract-N-Amp tissue PCR kit (Sigma- XNAT2). Placenta tissues were collected in RLT buffer and RNA was extracted using RNAeasy Mini Kit (Qiagen – 74104). RNA was eluted and concentration was estimated using

Nanodrop ND1000 spectrophotometer. For phenotypic analyses of PKCλ/ι KO embryos we analyzed total 101 embryos from 11 litters (Table 2). For phenotypic analyses of *Prkci* KD embryos we analyzed 35 control embryos and 36 *Prkci* KD embryos from eight individual experiments (Table 3).

Collection and analyses of human placentae: First-trimester placentae were obtained via services from RCWIH biobank, Toronto. Normal term placentae (≥38 week of gestation) were collected from Cesarean delivery at the KUMC. For PKCλ/ι expression analyses, 8 first-trimester placentae (6th-9th weeks of gestation) were sectioned and immunostained. Additionally, 6 normal term placentae were used for expression analyses.

Human TSC culture: Human TSC lines, derived from first trimester CTBs were described earlier (Okae et al., 2018). Although multiple lines were used, the data presented in this manuscript were generated using CT27 human TSC line. Human TSCs were cultured in DMEM/F12 supplemented with HEPES and L-glutamine along with cocktail of inhibitors. We followed the established protocol by Dr. Arima's group and induced SynT differentiation using Forskolin. Both male and female human TSC lines were used for initial experimentation. To generate *PRKCI* KD human TSCs, shRNA-mediated RNAi were performed. For initial screening, both male and female cell lines were used. As we did not notice any phenotypic difference after PRKCI-depletion, a female *PRKCI* KD human TSC line and corresponding control set were used for subsequent experimentation.

Trophectoderm-specific *Prkci* knockdown: Morula from day 2.5 plugged CD-1 superovulated females were treated with Acidic Tyrode's solution for removal of zona pellucida. The embryos were immediately transferred to EmbryoMax Advanced KSOM media. Embryos were treated with viral particles having either control pLKO.3G empty vector or shRNA against *Prkci* for 5 hours.

The embryos were washed 2-3 times, subsequently incubated overnight in EmbryoMax Advanced KSOM media and transferred into day 0.5 pseudopregnant females the following day. Uterine horns of control and knockdown sets were harvested at day 9.5. Placental tissue was obtained for RNA preparation to validate knockdown efficiency. Embryos were either dissected or kept frozen for sectioning purpose.

Transplantation of human TSCs into NSG mice: For transplantation analyses in NSG mice, Control and *PRKCI* KD human TSCs were mixed with matrigel and used to inject subcutaneously into the flank of NSG mice (6-9-week-old). 10^7cells were used for each transplantation experiment. Mice were euthanized after 7 days and trophoblastic lesions generated were isolated, photographed, measured for size, fixed, embedded in paraffin and sectioned for further analyses. Three individual experiments were performed and six mice (3 for control human TSCs and 3 for *PRKCI* KD human TSCs) were used in each experiment.

Mouse TSC culture: Mouse TSCs were cultured using RPMI-1640 (Sigma) supplemented with 20% Fetal Bovine Serum (FBS), sodium pyruvate, β-mercaptoethanol and primocin (to avoid any mycoplasma contamination). Proliferative cells were maintained using mouse embryonic fibroblast (MEF)-conditioned media (CM) and basal media (70:30 ratio), FGF4 (25ng/ml) and heparin (1µg/ml). For differentiation assays, cells were allowed to differentiate by removing CM, FGF4 and heparin from the media. Cells were harvested at different time points and total RNA and protein were extracted for RT-PCR and western blot analyses.

Human placental tissue sample analyses: Fresh and Formaldehyde fixed, de-identified first trimester placental tissues were obtained from Mount-Sinai hospital, Toronto. Term Placental tissues were obtained at the University of Kansas Medical Center with consent from patients. All collections and studies were approved by the University of Kansas IRB and the IRB at Mount

Sinai Hospital. Fresh term placental tissues were embedded in OCT and cryo-sectioned. Formaldehyde fixed and frozen sections were analyzed by immunohistochemistry.

Laser Capture Micro dissection and analyses of micro-dissected tissues: Tumors generated from transplantation of control and *PRKCI* KD HTS cells were stained for cytokeratin 7 and dissected out using laser capture microdissection (ZEISS Palm Microbeam). Term CTBs/SynTs were also dissected out using the same protocol. RNA was prepared using Arcturus Picopure RNA Isolation kit (Applied Biosystems-12204-01). cDNA was prepared using the entire RNA and amplification of the cDNA was performed using GenomePlex Complete Whole Genome Amplification (WGA) kit (Sigma-WGA2). The sample was purified using QIAquick PCR purification kit (Qiagen- 28104). Concentration of the DNA was measured using Nanodrop ND1000 Spectrophotometer.

mRNA expression analyses: Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen-74104) using manufacturer's protocol. cDNA was prepared from total RNA (1000ng). Primer cocktail comprising of 200ng/µl oligo dT and 50ng/µl random hexamer was annealed to the RNA at 68° for 10 minutes, followed by incubation with the master mix comprising of 5X first strand buffer, 10mM dNTPs, 0.1M DTT, RNase Inhibitor and M-MLV transcriptase (200U/µl) at 42° for 1 hour. The cDNA solution was diluted to 10ng/µl and heat inactivated at $95\ ^{O}$ for 5 minutes. Real-time PCR was performed using oligonucleotides (listed below). 20ng equivalent of cDNA was used for amplification reaction using Power SYBR Green PCR master mix (Applied Biosystems-4367659).

Western blot analyses: Cell pellets were washed once with 1X PBS followed by addition of 1X Laemmli SDS-PAGE buffer for protein extract preparation and western blot analyses were

performed following earlier described protocol (Saha et al., 2013). Antibodies used for the study are mentioned below.

RNA interference with mouse and human TSCs: Lentiviral-mediated shRNA delivery approach was used for RNAi. For mouse cells, shRNA was designed to target the 3' UTR sequence 5'-GTCGCTCTCGGTATCCTGTC-3' of the mouse *Prkci* gene. For control, a scramble shRNA (Addgene-1864) targeting a random sequence 5'-CCTAAGGTTAAGTCGCCCTCGC-3' was used. For human cells, short-hairpin RNA against *PRKCI* target sequence 5'-AGTACTGTTGGTTCGATTAAACTCGAGTTTAATCGAACCAACAGTACT-3' was used to generate lentiviral particles (Sigma Mission-TRCN0000219727). Lentiviral particles were generated by transfecting HEK293T cells. Earlier described protocols (P. Home et al., 2009) were followed to collect and concentrate viral particles. Mouse and human TSCs were transduced using viral particles with equal MOI at 60-70% confluency. The cells were treated with 8µg/ml polybrene prior to transduction. Cells were selected in the presence of puromycin (1.5-2µg/ml). Selected cells were tested for knockdown efficiency and used for further analyses. Freshly knocked-down cells were used for each individual experimental set to avoid any silencing of shRNA expression due to DNA-methylation at LTR. To generate data at least four individual experiments were done to get statistically significant results.

Immunofluorescence and Immunohistochemistry analyses: Immunofluorescence was performed using 10µm embryo cryosections. The sections were fixed using 4% paraformaldehyde in 1X PBS, permeabilized using 0.25% Triton X-100 in 1X PBS and blocked for 1 hour using 10% Normal Goat serum (Thermo Fisher scientific- 50062Z). The details of antibodies used are listed below. Immunohistochemistry was performed using paraffin sections of human placenta. The slides were deparaffinized by histoclear and subsequently with 100%, 90%, 80% and 70% ethanol.

Antigen retrieval was done using Decloaking chamber at 80°C for 15minutes. The slides were washed with 1X PBS and treated with 3% H_2O_2 to remove endogenous peroxidase followed by 3 times wash with 1X PBS. 10% goat serum was used as a blocking reagent for 1hour at RT followed by overnight incubation with 1:100 dilution of primary antibody or IgG at 4°C. The slides were washed with 1X PBS and 1:200 dilution of secondary antibody was used for 1 hour at RT. The slides were washed again with 1X PBS followed by treatment with horseradish peroxidase streptavidin for 20 minutes at RT. The slides were washed again and proceeded to color development using DACO 1ml buffer and 1 drop of chromogen. The reaction was stopped in distilled water after sufficient color developed. The slides were counterstained with Mayer's hematoxylin for 5minutes and washed with warm tap water until sufficient bluish coloration observed. The slides were then dehydrated by sequential treatment using 70%, 80%, 90%, 100% ethanol and histoclear. The sections were completely dried and mounted using Toluene as mountant and imaged using Nikon TE2000 microscope.

Cell proliferation assay: Mouse and Human TSCs were seeded (30,000cells/well of 12 well plate) and cultured for 24, 48, 72, 96 hours to assess cell proliferation. Cell proliferation was assessed using both BrDU labeling assay and detection kit (Roche Ref#11296736001) in live cells and with MTT assay kit (Catalog# Sigma CGD1) after harvesting cells at distinct time intervals. We followed manufacturers' protocol.

RNA-In Situ hybridization (RNA-ISH): RNA-ISH was performed using ACDBio RNASCOPE kit (Catalog # 320851 and 322310) and following manufacturer's protocol. The probes for mouse *Prkci* (Catalog#403191), *Dlx3* (Catalog#425191), *Syna* (Catalog#446701), *Gcm1* (Catalog#429661), *Tpbpa* (Catalog#405511) and human *GATA2* (Catalog#414361) and *GCM1* (Catalog#429711) were custom designed by ACDbio.

Testing HCG secretion from Human TSCs: Cell supernatants were collected from control and *PRKCI* KD HTS at Day5 of differentiation towards syncytiotrophoblast. ELISA kit was used from Sigma (RAB0092-1KT) and manufacturer's protocol was followed.

RNA-seq analyses: Total RNA was used to construct RNA-seq libraries using the Illumina TruSeq Stranded Total RNA Sample Preparation Kit according to manufacturer's instructions. RNA seq was performed using Illumina HiSeq 2500 platform. Raw sequence reads in fastq format were mapped to the *Mus musculus* GRCm38 genome with STAR-2.5.2b (Dobin et al., 2013). Gene expression levels were estimated with StringTie-1.3.0 (Pertea et al., 2015) and cuffmerge (Trapnell et al., 2012) from the cufflinks2 (Anders, Pyl, & Huber, 2015) package. Gene counts were obtained with htseq-0.6.1p1 followed by differential gene expression analysis with EdgeR (Robinson, McCarthy, & Smyth, 2010). The data has been deposited and is available in the GEO database (Accession number- GSE100285).

Statistical significance: Statistical significances were determined for quantitative RT-PCR analyses, analyses of SynT-differentiation efficiency and HCG secretion by Human TSCs. We have performed at least n=3 experimental replicates for all those experiments. For statistical significance of generated data, statistical comparisons between two means were determined with Student's t-test. Although in few figures studies from multiple groups are presented, the statistical significance was tested by comparing data of two groups and significantly altered values ($p \leq 0.05$) are highlighted in figures by an asterisk. RNA-Seq data were generated with n=3 experimental replicates per group. The statistical significance of altered gene expression (2-fold change) was initially confirmed with right tailed Fisher's exact test with p-value cutoff set at 0.01. The final list of altered genes that were presented in Figure 12C was selected with an additional FDR cutoff set at 0.1.

Oligomers Used for The Study

Genotyping primers:

Gene	Forward	Reverse
Prkci wildtype	*AACACCAGGGAGAGTGG*	*GCCTAGAAAGTAACCCAC*
Prkci knockout	*AACACCAGGGAGAGTGG*	*GACGAGTTCTTCTGAGGGG*

RT-PCR mouse primers:

Gene	Forward	Reverse
Prkci Ex9	*CTAGGTCTGCAGGATTTCG*	*TTGACGAGCTCTTTCTTCAC*
Prkci (Ex1/2)	*TCCGGGTGAAAGCCTACTAC*	*CAAAGGAGATGGAAGGCTCA*
Prkcz	*GGACAACCCTGACATGAACAC*	*GGCCTTGACAGACAGGAAAC*
18SrRNA	*AGTTCCAGCACATTTTGCGAG*	*TCATCCTCCGTGAGTTCTCCA*
Cdx2	*GGACGTGAGCATGTATCCTAGCT*	*TAACCACCGTAGTCCGGGTACT*
Eomes	*ACCAATAACAAAGGTGCAAACAAC*	*TGGTATTTGTGCAGAGACTGCAA*
Esrrb	*AGTACAAGCGACGGCTGG*	*CCTAGTAGATTCGAGACGATCTTAGTCA*
Elf5	*ATGTTGGACTCCGTAACCCAT*	*GCAGGGTAGTAGTCTTCATTGCT*
Gata3	*CGGGTTCGGATGTAAGTCGA*	*GTAGAGGTTGCCCCGCAGT*
Gcm1	*AGAGATACTGAGCTGGGACATT*	*CTGTCGTCCGAGCTGTAGATG*
Dlx3	*CACTGACCTGGGCTATTACAGC*	*GAGATTGAACTGGTGGTGGTAG*
Tead4	*ATCCTGACGGAGGAAGGCA*	*GCTTGATATGGCGTGCGAT*
Gata2	*GGAAGATGTCCAGCAAATCC*	*TGGAGAGCTCCTCGAAACAT*
Pparg	*AGCTGTCATTATTCTCAGTGGAG*	*ATGTCCTCGATGGGCTTCAC*
Ascl2	*AAGCACACCTTGACTGGTACG*	*AAGTGGACGTTTGCACCTTCA*
Hand1	*CTACCAGTTACATCGCCTACTTG*	*ACCACCATCCGTCTTTTTGAG*
Cx31	*TCTGGCTGTCAGTAGTGTTCG*	*GCCTGGTGTTACAGTCAAAGTC*
Tpbpa	*TCCGGTCAGCTAACTGATGA*	*TCCTCTTCAAACATTGGGTGT*
Prl3d1	*ACATTTATCTTGGCCGCAGATGTGT*	*TTTAGTTTCGTGGACTTCCTCTCGAT*

RT-PCR human primers:

Gene	Forward	Reverse
PRKCI	*AGGTCCGGGTGAAAGCCTA*	*TGAAGAGCTGTTCGTTGTCAAA*
PRKCZ	*ATGACGAGGATATTGACTGGGT*	*CAGGAGTGTAATCCGACCAGG*
GATA2	*CCAGCTTCACCCCTAAGCAG*	*CCACAGTTGACACACTCCCG*
PPARG	*ACCAAAGTGCAATCAAAGTGGA*	*ATGAGGGAGTTGGAAGGCTCT*
GCM1	*GGCGCAAGATCTACCTGAGA*	*CACAGTTGGGACAGCGTTT*
ERVW-1	*CTACCCCAACTGCGGTTAAA*	*GGTTCCTTTGGCAGTATCCA*
CGA	*TCTGGTCACATTGTCGGTGT*	*TTCCTGTAGCGTGCATTCTG*
CGB	*GTGTGCATCACCGTCAACAC*	*GGTAGTTGCACACCACCTGA*
PSG4	*CGATGGGACTGGAGGAGTAA*	*AGTTGCTGCTGGAGATGGAG*
HPRT1	*ACCCTTTCCAAATCCTCAGC*	*GTTATGGCGACCCGCAG*
18SrRNA	*AACCCGTTGAACCCCATT*	*CCATCCAATCGGTAGTAGCG*
CDX2	*CTGGTTTCAGAACCGCAGAG*	*TGCTGCTGCAACTTCTTCTT*
ELF5	*CTGCCTTTGAGCATCAGACA*	*TCCAGTATTCAGGGTGGACTG*
TEAD4	*ACGGCCTTCCACAGTAGCAT*	*CTTGCCAAAACCCTGAGACT*

Antibodies Used for the Study

List of primary antibodies:

Name	Company	Catalog#
PKCiota	*BD Transduction Laboratories*	*610175*
Pan-cytokeratin	*Abcam*	*ab9377*
CDX2	*Abcam*	*ab76541*
β-actin	*Sigma*	*A5441*
HCGβ	*Abcam*	*ab53087*
Cytokeratin 7	*Dako*	*M7018*
E-cadherin	*Abcam*	*ab1416*
PPARγ (mouse)	*Proteintech*	*16643-1-AP*

PPARγ (human)	*SantaCruz*	*sc81152*
GATA2	*Abcam*	*ab109241*
MCT1	*EMD Millipore*	*AB1286-I*
MCT4	*EMD Millipore*	*AB3314P*
Proliferin	*Santa Cruz*	*sc-47347*

List of secondary antibodies:

Name	**Company**	**Catalog#**
Alexa fluor 488 goat anti-rabbit IgG	*Invitrogen*	*A11008*
Alexa fluor 568 goat anti-mouse IgG	*Invitrogen*	*A11031*
Alexa fluor 488 donkey anti-mouse IgG	*Invitrogen*	*A21202*
Alexa fluor 568 donkey anti-rabbit IgG	*Invitrogen*	*A10042*
Alexa fluor 568 donkey anti-goat IgG	*Invitrogen*	*A11057*
Alexa fluor 488 goat anti-chicken IgY	*Invitrogen*	*A32931*
Goat anti-mouse IgG-HRP	*Santa Cruz*	*sc2005*
Goat anti-rabbit IgG-HRP	*Santa Cruz*	*sc2004*

RESULTS

PKCλ/ι protein expression is selectively abundant in the trophoblast progenitors of developing mouse and human placentae.

Earlier studies showed that PKCλ/ι is ubiquitously expressed in all cells of a developing pre-implantation mouse embryo (Saiz, Grabarek, Sabherwal, Papalopulu, & Plusa, 2013), including the trophectoderm cells. Also, another study showed that PKCλ/ι is ubiquitously expressed in both embryonic and extraembryonic cell lineages in a postimplantation E7.5 mouse embryo (Seidl et al., 2013). We validated both observations in a blastocyst (Figure 1A) and in E7.5 mouse embryo (Figure 1B). However, relative abundance of PKCλ/ι protein expression in different cell types is not well documented during postimplantation mouse development. Therefore, we tested PKCλ/ι protein expression at different stages of mouse post-implantation development. We found that in ~E8 mouse embryos, PKCλ/ι protein is most abundantly expressed in the TSPCs residing in the placenta primordium (Figure 1C). In comparison, cells within the developing embryo proper showed very low levels of PKCλ/ι protein expression. The high abundance of *Prkci* mRNA and PKCλ/ι protein expression was detected in the trophoblast cells of an E9.5 mouse embryo (Figure 2A and 2B). We also observed that PKCλ/ι is expressed in the maternal decidua. Surprisingly, the embryo proper showed extremely low PKCλ/ι protein expression at this developmental stage. The abundance of PKCλ/ι protein expression in the trophoblast is also maintained as development progresses. However, cells in the embryo proper also show PKCλ/ι protein expression beginning at mid-gestation as in E12.5 (Figure 2C).

As PKCλ/ι expression during human placentation has never been tested, we investigated PKCλ/ι protein expression in human placentae at different stages of gestation. Our analyses revealed that PKCλ/ι protein is expressed specifically in the villous CTBs within a first-trimester

human placenta (Figure 3A). PKCλ/ι is also expressed in CTBs within term placenta (Figure 3B). To further quantitate PKCλ/ι expression, we isolated CTBs from first trimester and term placentae and analyzed *PRKCI* mRNA expression. We also isolated SynT from term placentae via laser capture microdissection (LCM). We found that *PRKCI* mRNA expression is ~ 2-fold higher in first trimester CTBs compared to that in term CTBs. However, PKCλ/ι expression is strongly repressed in differentiated SynTs in first-trimester human placentae as well as in term-placentae (Figure 3C).

Global Loss of PKCλ/ι in a developing mouse embryo abrogates placentation.

Global deletion of *Prkci* in a developing mouse embryo (PKCλ/ι KO embryo) results in gastrulation defect leading to embryonic lethality (Seidl et al., 2013; Soloff et al., 2004) at ~E9.5. However, the placentation process was never studied in PKCλ/ι KO mouse embryos. As many embryonic lethal mouse mutants are associated with placentation defects, we probed into trophoblast development and placentation in post-implantation PKCλ/ι KO embryos. We set up matings using the heterozygous animals and analyzed embryos at different gestational ages (Figure 4A). We started investigating placenta and trophoblast development in PKCλ/ι KO embryos starting from E7.5. At this stage the placenta primordium consists of the ExE/EPC regions. However, we did not notice any obvious phenotypic differences of the ExE/EPC development between the control and the PKCλ/ι KO embryos at E7.5 (Figure 4B). We noticed developmental defect in PKCλ/ι KO placentae after the chorio-allantoic attachment, an event which takes place at ~ E8.5. We noticed that PKCλ/ι KO developing placentae were smaller in size at E8.5 (Figure 4C) and this defect in placentation was more prominent in E9.5 embryos. At E9.5, the placentae in PKCλ/ι KO embryos were significantly smaller in size (Figure 4D) and the embryo proper showed gross impairment in development due to defective gastrulation (Figure 4E), as reported in

earlier studies (Soloff et al., 2004). Immunofluorescence analyses revealed defective embryonic-extraembryonic attachment with an altered orientation of the embryo proper with respect to the developing placenta (Figure 5A).

We also failed to detect any visible labyrinth zone in most of the PKCλ/ι KO placentae. The abrogation of the placental labyrinth and the SynT development were confirmed by a near complete absence of *Gcm1* mRNA expression (Figure 6A) and lack of any *Dlx3*-expressing labyrinth trophoblast cells in E9.5 PKCλ/ι KO placentae (Figure 6B). In contrast, the PKCλ/ι KO placentae mainly contained proliferin (PLF) expressing trophoblast giant cells (TGCs) (Figure 5B).

In a very few PKCλ/ι KO placentae (3 out of 32 analyzed by sectioning), other trophoblast cells exist along with TGCs (Figure 5B, red border). We tested presence of different trophoblast subtypes in those placentae. We detected trophoblast-specific protein alpha (*Tpbpa)-* expressing spongiotrophoblast population (Figure 5C). We also tested for labyrinth markers. In a mouse placenta the matured labyrinth contains two layers of SynTs, SynT-I and SynT-II. The SynT-I cells express the retroviral gene syncytin A (SynA) (Dupressoir et al., 2009), whereas the SynT-II population arises from *Gcm1* expressing progenitors (D. G. Simmons et al., 2008). So, we tested presence of those cells via in situ hybridization (ISH). We detected presence of some *SynA*-expressing cells (Figure 5C). However, we could not detect any *Gcm1* expressing cells in any of the PKCλ/ι KO placenta (Figure 6A). We further tested formation of SynT-I and SynT-II layers by analyzing expressions of Solute Carrier Family 16 Member 1 (MCT1) and Solute Carrier Family 16 Member 3 (MCT4), respectively (Nagai, Takebe, Nio-Kobayashi, Takahashi-Iwanaga, & Iwanaga, 2010). In contrast to control placentae, in which we detected development of two MCT1 and MCT4 expressing SynT layers (Figure 6C), we only detected a few MCT1 expressing

cells in PKCλ/ι KO placentae. However, those cells were dispersed, indicating lack of cell fusion and formation of a matured SynT-I layer. We could not detect any MCT4 expressing SynT-II cells in PKCλ/ι KO placentae. These results indicated that loss of PKCλ/ι leads to abrogation of SynT-II development. Although a few SynA/MCT1 expressing SynT-I like population could arise in a few PKCλ/ι KO placentae, they do not differentiate to a matured SynT-I layer (Figure 6C).

We also isolated RNA from placental tissue and analyzed the expression of *Gcm1* and *Dlx3*. We observed impaired transcriptional induction of both *Gcm1* and *Dlx3* in E8.5 PKCλ/ι KO placentae compared to control. However, *Gcm1* and *Dlx3* is expressed at very low levels in E7.5 placenta, both in the control and PKCλ/ι KO embryos (Figure 7A). We also observed loss of *Gcm1* expression in E9.5 PKCλ/ι KO placenta compared to control (Figure 7B). We could not test placentation in the PKCλ/ι KO embryos beyond E9.5 as these embryos and placentae begin to resorb at late gestational stages. Thus, from our findings, we concluded that the global loss of PKCλ/ι in a developing mouse embryo leads to defective placentation after the chorio-allantoic attachment due to impaired development of the SynT lineage, resulting in abrogation of placental labyrinth formation. The total number of embryos analyzed in each set is indicated in Table1 and 2.

Trophoblast-specific PKCλ/ι depletion impairs mouse placentation leading to embryonic death.

Since we observed placentation defect in the global PKCλ/ι KO embryos, we next interrogated the importance of trophoblast cell specific PKCλ/ι function in mouse placentation and embryonic development. Although a *Prkci*-conditional knockout mouse model exists, we could not get access to that mouse. Therefore, we performed RNAi using lentiviral mediated gene delivery approach as described earlier (D. S. Lee, Rumi, Konno, & Soares, 2009) to specifically deplete PKCλ/ι in

the developing trophoblast cell lineage. We transduced zona-removed mouse blastocysts with lentiviral particles with shRNA against *Prkci* (Figure 8A) and transferred them to pseudo pregnant females. We confirmed the efficiency of shRNA-mediated PKCλ/ι-depletion by measuring *Prkci* mRNA expression in transduced blastocysts (Figure 8B) and also by testing loss of PKCλ/ι protein expressions in trophoblast cells of developing placentae in multiple experiments (Figure 8C). Intriguingly, the trophoblast specific PKCλ/ι depletion also resulted in embryonic death before E9.5 due to severe defect in the placenta and embryo proper (Figure 8D). Furthermore, the immunofluorescence analyses of trophoblast cells at ~E9.5 confirmed defective placentation in the *Prkci* KD placentae, characterized with a near complete absence of the labyrinth zone (Figure 9A) and *Dlx3* expressing SynT populations (Figure 9B). However, similar to PKCλ/-KO placentae, *Prkci* KD placentae predominantly contained PLF-expressing TGC populations (Figure 9C). Thus, the trophoblast-specific depletion of PKCλ/ι in a developing mouse embryo recapitulated similar placentation defect and embryonic death as observed in the global PKCλ/ι KO embryos. Table 3 indicates number of *Prkci* KD embryos analyzed.

PKCλ/ι signaling in a developing mouse embryo is essential to establish a transcriptional program for TSPC to SynT differentiation.

The abrogation of labyrinth development in the trophoblast specific *Prkci* KD mouse placentae indicated a critical importance of the PKCλ/ι signaling in SynT development and labyrinth formation. During mouse placentation, the SynT differentiation is associated with the suppression of TSC/TSPC-specific genes, such as Caudal type homeobox 2 (*Cdx2)*, Eomesodermin *(Eomes),* TEA domain transcription factor 4 *(Tead4),* Estrogen related receptor beta *(Esrrβ)* and E74 like transcription factor 5 (*Elf5)* (Donnison et al., 2005; P. Home et al., 2012; Latos et al., 2015; R. Michael Roberts & Susan J. Fisher, 2011; Russ et al., 2000; D. Strumpf et al., 2005) and induction

of expression of the SynT-specific genes, such as *Gcm1, Dlx3* and fusogenic retroviral genes SyncytinA and SyncytinB (Janet Rossant & James C. Cross, 2001). In addition, other transcription factors, such as PPARγ, GATA transcription factors- GATA2 and GATA3, and cell signaling regulators, including members of mitogen activated protein kinase pathway are implicated in mouse SynT development (P. Home et al., 2017; Nadeau et al., 2009; Mana M. Parast et al., 2009). Therefore, to define the molecular mechanisms of PKCλ/ι-mediated regulation of SynT development, we specifically depleted PKCλ/ι expression in mouse TSCs via RNAi (Figure 10A) and asked whether the loss of PKCλ/ι impairs mouse TSC self-renewal or their differentiation to specialized trophoblast cell types.

When cultured in stem-state culture condition (with FGF4 and Heparin), PKCλ/ι-depleted mouse TSCs (*Prkci* KD mouse TSCs) did not show any defect in the stem-state colony morphology (Figure 10B). Also, cell proliferation analyses by MTT assay and BrDU incorporation assay indicated that cell proliferation was not affected in the *Prkci* KD mouse TSCs (Figure 10C and 10D). Furthermore, mRNA expression analyses showed that expression of TSC stem-state regulators, such as *Cdx2, Eomes, Gata3, Tead4, Esrrb and Elf5* were not affected upon PKCλ/ι depletion (Figure 11A). Western blot analysis also confirmed that CDX2 protein expression was not affected in the *Prkci* KD TSCs (Figure 11B). These results indicated that PKCλ/ι signaling is not essential to maintain the self-renewal program in mouse TSCs.

Next, we asked whether the loss of PKCλ/ι affects mouse TSC differentiation program. Removal of FGF4 and heparin from the culture medium induces spontaneous differentiation in mouse TSCs, which can be monitored over a course 6-8 days. During this differentiation program, induction of SynT-differentiation markers like *Gcm1*, *Dlx3* can be monitored in differentiating cells between day 2 - day 4. Subsequently, the TSC markers are repressed in the differentiating

TSCs as the TGC-specific differentiation program becomes more prominent. Thus, after day 6 of differentiation, mouse TSCs highly express TGC specific markers, like Prolactin family 3 subfamily d member 1 (*Prl3d1),* Heart and neural crest-derived transcript 1 *(Hand1),* Prolactin family 2 subfamily c member 2 *(Prl2c2)*. In addition, Trophoblast specific protein alpha (*Tpbpa),* Achaete-scute homologue 2 *(Ascl2),* Connexin 31 *(Cx31)*, which are markers of spongiotrophoblast and glycogen trophoblast cells of the placental junctional zone, are also induced in differentiated mouse TSCs.

As the loss of PKCλ/ι affects labyrinth development, we monitored expressions of *Gcm1 and Dlx3* in differentiating *Prkci* KD mouse TSCs. Similar to our findings with the PKCλ/ι-depleted placentae, induction of *Dlx3* and *Gcm1* mRNA expression was impaired in differentiating *Prkci* KD mouse TSCs (Figure 11D). In contrast, induction of *Tpbpa, Prl3d1, Cx31*, which are markers for spongiotrophoblasts, TGCs and glycogen cells, respectively, were not affected in differentiated *Prkci* KD mouse TSCs (Figure 11C). Thus, we concluded that the loss of PKCλ/ι in mouse TSCs does not affect their differentiation to specialized trophoblast cells of the placental junctional zone, such as spongiotrophoblasts, TGCs, and the glycogen cells. Rather, PKCλ/ι is essential to specifically establish the SynT differentiation program in mouse TSCs.

Based on our findings with *Prkci* KD mouse TSCs, we hypothesized that the PKCλ/ι signaling might regulate key genes, which are specifically required to induce the SynT differentiation program in TSCs. To test this hypothesis, we performed unbiased whole RNA sequencing (RNA-seq) analysis with *Prkci* KD mouse TSCs. RNA-seq analyses showed that the depletion of PKCλ/ι in mouse TSCs altered expression of 164 genes by at least two folds with a high significance level ($p \leq 0.01$). Among these 164 genes, 120 genes were downregulated, and 44 genes were upregulated (Figure 12A). Ingenuity pathway analyses revealed multi-modal

biofunctions of PKCλ/ι regulated genes, including involvement in embryonic and reproductive developments (Figure 12B). To further gain confidence on PKCλ/ι regulated genes in the mouse TSCs, we curated the number of altered genes with a false discovery rate (FDR) threshold of 0.1. The FDR filtering identified only 6 upregulated genes and 46 downregulated genes in the *Prkci* KD mouse TSCs (Figure 12C and 12D). Among the downregulated genes, *Prkci* was identified as the most significantly altered gene, thereby confirming the specificity and high efficiency of the shRNA mediated *Prkci* depletion.

Among the six genes, which were significantly upregulated in *Prkci* KD mouse TSCs, only Growth differentiation factor 6 *(Gdf6)* has been implicated in trophoblast biology in an overexpression experiment with embryonic stem cells (Lichtner, Knaus, Lehrach, & Adjaye, 2013). However, *Gdf6* deletion in a mouse embryo does not affect placentation (Clendenning & Mortlock, 2012; Mikic, Rossmeier, & Bierwert, 2009). In contrast, three transcription factors, *Gata2, Pparg* and *Cited2,* which were significantly downregulated in the *Prkci* KD mouse TSCs, are implicated in the regulation of trophoblast differentiation and labyrinth development. Earlier gene knockout studies implicated CITED2 in the placental labyrinth formation. However, CITED2 is proposed to have a non-cell autonomous role in SynT as its function is more important in proper patterning of embryonic capillaries in the labyrinth zone rather than in promoting the SynT differentiation (Withington et al., 2006). In contrast, knockout studies in mouse TSCs indicated that PPARγ is an important regulator for SynT differentiation (Mana M. Parast et al., 2009). PPARγ-null mouse TSCs showed specific defects in SynT differentiation and rescue of PPARγ expression rescued *Gcm1* expression and SynT differentiation. Also, earlier we showed that in mouse TSCs, GATA2 directly regulates expression of several SynT-associated genes including *Gcm1* and, in coordination with GATA3, ensures placental labyrinth development (P. Home et al.,

2017). Therefore, we focused our study on GATA2 and PPARγ and further tested their expressions in *Prkci* KD mouse TSCs and PKCλ/ι KO placenta primordium. We validated the loss of GATA2 and PPARγ protein expressions in *Prkci* KD mouse TSCs (Figure 13A). Our analyses confirmed that both *Gata2* and *Pparg* mRNA expression are significantly downregulated in E7.5 PKCλ/ι KO placenta primordium (Figure 13B) and the loss of *Gata2* and *Pparg* expression was subsequently associated with impaired transcriptional induction of both *Gcm1 and Dlx3* in E8.5 PKCλ/ι KO placentae (Figure 7A). Thus, our studies in *Prkci* KD mouse TSCs and PKCλ/ι KO placenta primordium indicated a regulatory pathway, in which the PKCλ/ι signaling in differentiating TSPCs ensures GATA2 and PPARγ expression, which in turn establish proper transcriptional program for SynT differentiation (Figure 13C).

PKCλ/ι is critical for human trophoblast progenitors to undergo differentiation towards syncytiotrophoblast lineage.

Our expression analyses revealed that PKCλ/ι expression is conserved in CTB progenitors of a first-trimester human placenta. However, functional importance of PKCλ/ι in the context of human trophoblast development and function has never been tested. We wanted to test whether PKCλ/ι signaling mediates a conserved function in human CTB progenitors to induce SynT differentiation. However, testing molecular mechanisms in isolated primary first trimester CTBs is challenging due to lack of established culture conditions, which could maintain CTBs in a self-renewing stage or could promote their differentiation to SynT lineage. Rather, the recent success of derivation of human trophoblast stem cells (human TSCs) from first-trimester CTBs (Okae et al., 2018) has opened up new opportunities to define molecular mechanisms that control human trophoblast lineage development. When grown in media containing a Wnt activator CHIR99021, EGF, Y27632 (a Rho-associated protein kinase [ROCK] inhibitor), A83-01, and SB431542 (TGFβ

inhibitors) and valproic acid (VPA) (a histone deacetylase [HDAC] inhibitor), the established human TSCs can be maintained in a self-renewing stem state for multiple passages. In contrast, when cultured in the presence of cAMP agonist forskolin, human TSCs synchronously differentiate and fuse to form two-dimensional (2D) syncytia on a high-attachment culture plate or three-dimensional cyst-like structures on a low adhesion culture plate. In both 2D and 3D culture conditions, differentiated human TSCs highly express SynT markers and secrete a large amount of human chorionic gonadotropin (hCG). Thus, depending on the culture conditions, human TSCs efficiently recapitulate both the self-renewing CTB progenitor state and their differentiation to SynTs. Therefore, we used human TSCs as a model system and performed loss-of-function analyses to test importance of PKCλ/ι signaling in human TSC self-renewal vs. their differentiation towards the SynT lineage.

We performed lentiviral-mediated shRNA delivery to deplete PKCλ/ι expression in human TSCs (*PRKCI* KD human TSCs). The shRNA-mediated RNAi in human TSCs reduced *PRKCI* mRNA expression by more than 90% without affecting the *PRKCZ* mRNA expression (Figure 14A and 14B), thereby, confirming the specificity of the RNAi approach. Similar to *Prkci* KD mouse TSCs, *GATA2, PPARG* and *GCM1* mRNA expressions were significantly reduced in *PRKCI* KD human TSCs (Figure 14A). Western blot analyses also confirmed loss of GATA2 and PPARγ protein expressions in *PRKCI* KD human TSCs (Figure 14B). However, the loss of PKCλ/ι expression in human TSCs did not overtly affect their stem-state morphology (Figure 14C-14E), proliferation (Figure 15A and 15B) or expression of trophoblast stem state markers, such as *TEAD4 or CDX2* (Figure 15C). Rather, we observed a smaller (~25%) induction in *ELF5* mRNA expression upon loss of PKCλ/ι (Figure 15C). Thus, we concluded that PKCλ/ι signaling is not essential to maintain the self-renewing stem-state in human TSCs, rather it is important to maintain optimum expression

of key genes like *GATA2, PPARG* and *GCM1*, which are known regulators of SynT differentiation. Therefore, we next interrogated SynT differentiation efficiency in *PRKCI* KD human TSCs.

We cultured control and *PRKCI* KD human TSCs with forskolin on both high and low-adhesion culture plates to test the efficacy of both 2D and 3D syncytium formation. We assessed 2D SynT differentiation by monitoring elevated mRNA expressions of key SynT-associated genes, such as the HCGβ components *CGA*, *CGB;* retroviral fusogenic protein *ERVW1* and pregnancy-associated glycoprotein, *PSG4*. We also tested HCGβ protein expression and monitored cell syncytialization via loss of E-CADHERIN (CDH1) expression in fused cells. We found strong impairment of SynT differentiation of *PRKCI* KD human TSCs (Figure 16A-D). Unlike in control human TSCs, mRNA induction of key SynT-associated genes (Figure 16D) as well as HCGβ protein expression were strongly inhibited in *PRKCI* KD human TSCs. Furthermore, *PRKCI* KD human TSCs maintained strong expression of CDH1 and showed a near complete inhibition of cell-fusion (Figure 16B).

The impaired SynT differentiation potential in *PRKCI* KD human TSCs were also evident in the 3D culture conditions. Unlike control human TSCs, which efficiently formed large cyst-like spheres (larger than 50µm in diameter), *PRKCI* KD human TSCs failed to efficiently develop into larger spheres and mainly developed smaller cellular aggregates (Figure 17A and 17C). Also, comparative mRNA expression analyses indicated more abundance of *PRKCI* mRNA in a few larger spheres, which were developed with *PRKCI* KD human TSCs, indicating that the large spheres are formed from cells, in which RNAi-mediated gene depletion was inefficient. We noticed significant inhibition of *CGA, CGB, ERVW1* and *PSG4* mRNA inductions in the small cell aggregates, which also showed significant downregulation in *PRKCI* mRNA expressions (Figure 17B). We also confirmed loss of HCG secretion by *PRKCI* KD human TSCs by measuring HCG

concentration in the culture medium (Figure 17D). Thus, both the 2D and 3D SynT differentiation systems revealed impaired SynT differentiation potential of the *PRKCI* KD human TSCs.

PKCλ/ι signaling is essential for in vivo SynT differentiation potential of Human TSCs.

As discussed above, our in-vitro differentiation analysis indicated an important role of PKCλ/ι signaling in inducing SynT differentiation potential in the human TSCs. However, the *in vitro* differentiation system lacks the complex cellular environment and regulatory factors that control SynT development during placentation. Okae et al. showed that upon subcutaneous injection into non-diabetic-severe combined immunodeficiency mice (NOD-SCID mice), human TSCs invaded the dermal and underlying tissues to establish trophoblastic lesions (Okae et al., 2018). These trophoblastic lesions contain cells that represent all cell types of a villous human placenta, namely CTB, SynT and EVT. Therefore, we next tested *in vivo* SynT differentiation potential of *PRKCI* KD human TSCs via transplantation in the NOD-SCID mice (Figure 18A). Upon transplantation, both control and *PRKCI* KD human TSCs generated tumors with similar efficiency (Figure 18B) and the presence of human trophoblast cells were confirmed via analyses of human KRT7 expression (Figure 19A). However, unlike control human TSCs, lesions that were developed from *PRKCI* KD human TSCs, were largely devoid of HCGβ -expressing SynT populations (Figure 19A). We also confirmed loss of *GATA2* and *GCM1* mRNA expressions in lesions that were developed from *PRKCI* KD human TSCs (Figure 19B) and also from RNA obtained from laser capture microdissection of tumor sections (Figure 19C and 19D). Collectively, our *in vitro* differentiation and *in vivo* transplantation assays with human TSCs imply an essential and conserved molecular mechanism, in which the PKCλ/ι signaling promotes expression of key transcription factors, like GATA2, PPARγ and GCM1 to assure CTB to SynT differentiation.

FIGURES

Figure 1:

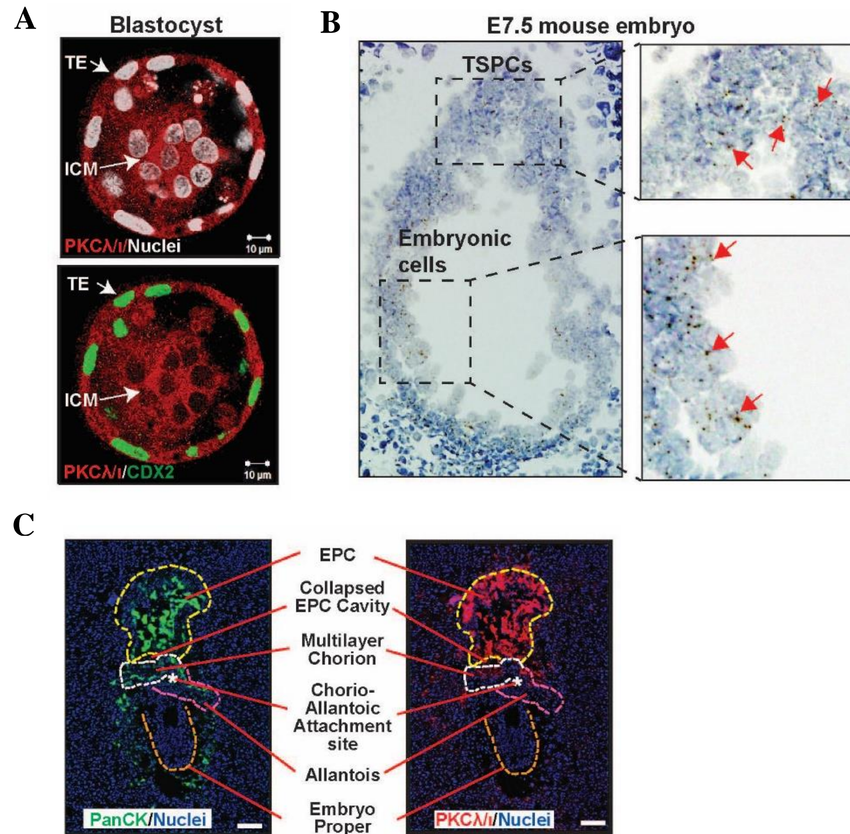


Figure 1: Expression analyses for PKCλ/ι mRNA and protein: (A) Immunofluorescence of mouse blastocyst using anti-PKCλ/ι antibody (red), anti-CDX2 (green) and DAPI. Images show that PKCλ/ι is expressed in both inner cell mass and in trophectoderm. (B) RNA in situ hybridization was performed using fluorescent probes against *Prkci* mRNA in E7.5 mouse implantation site. Images show expression of *Prkci* mRNA (red punctate dots marked by red arrows) in the embryonic cells as well in the TSPCs within placenta primordium. (C) Immunofluorescence images showing trophoblast progenitors, marked by anti-pancytokeratin antibody (green), expressing high levels of PKCλ/ι protein (red) in an ~E8 mouse embryo. Note much less expression of PKCλ/ι protein within cells of the developing embryo proper (orange dotted boundary). EPC, Chorion, allantois and the chorio-allantoic attachment site are indicated with yellow, white, pink dotted lines and a white asterisk, respectively. (Scale bars: 100 µm).

Figure 2:

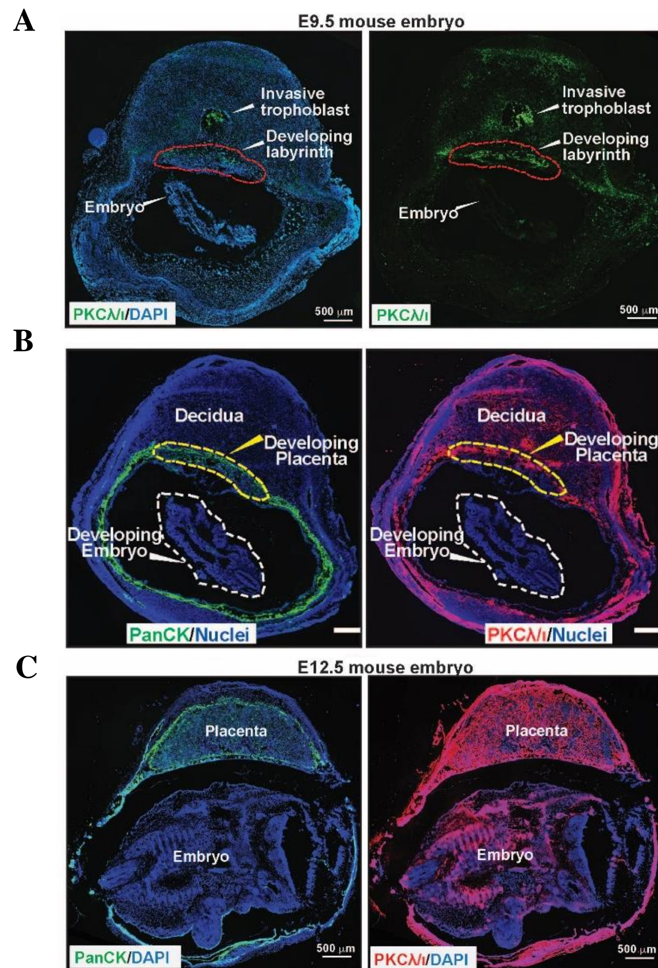


Figure 2: Expression analyses for PKCλ/ι mRNA and protein in mouse post-implantation embryos: (A) RNA in situ hybridization was performed in E9.5 mouse implantation site showing prominent *Prkci* mRNA expression in the labyrinth zone and maternal decidua. (B) Immunofluorescence images of an E9.5 mouse implantation sites showing pan-Cytokeratin (left), PKCλ/ι (right) and nuclei (DAPI). At this developmental stage, PKCλ/ι protein is highly expressed in trophoblast cells of the developing placenta and in maternal uterine cells. However, PKCλ/ι protein expression is much less in the developing embryo. (Scale bars: 500 µm). (C) Immunofluorescence of E12.5 mouse embryo using anti-pan-cytokeratin (PanCK, trophoblast marker) antibody (left panel) and anti-PKCλ/ι antibody (right panel). Nuclei were stained with DAPI. At this developmental stage, PKCλ/ι protein expression is prominent in both placenta and in the embryo proper.

Figure 3:

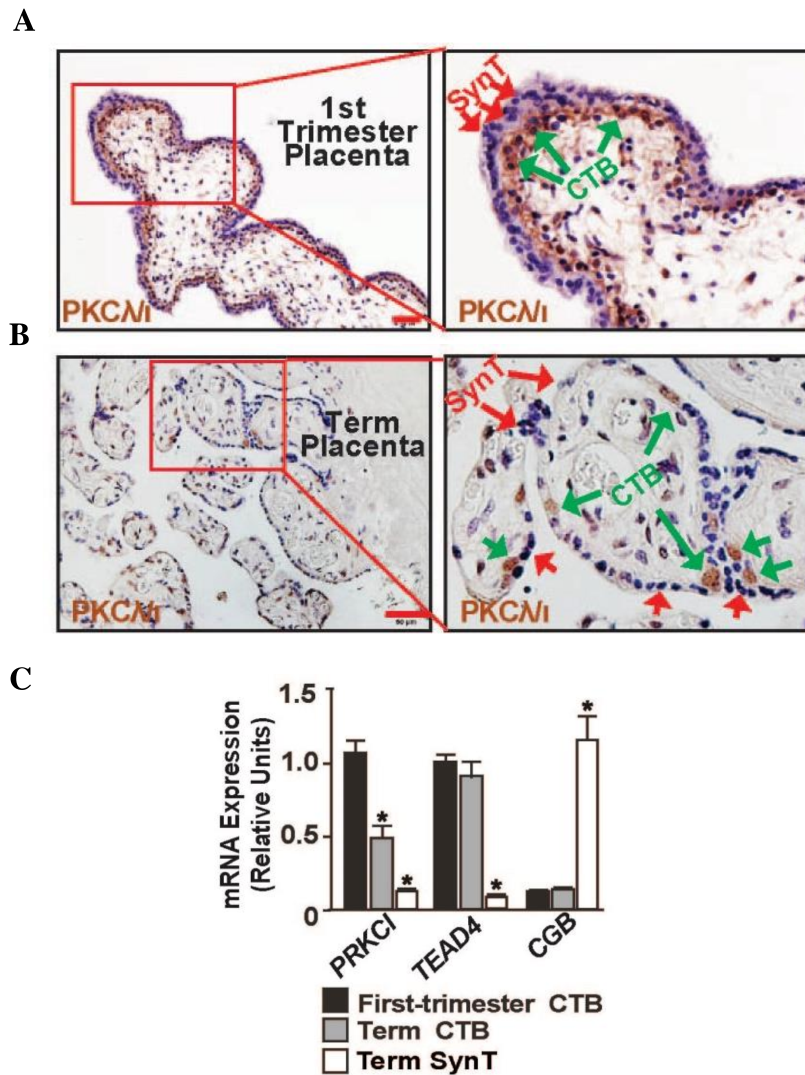


Figure 3: Expression analyses for PKCλ/ι mRNA and protein in human first trimester and term placenta: (A) Immunohistochemistry showing PKCλ/ι is selectively expressed within the cytotrophoblast progenitors (green arrows) of first trimester (8 week) and (B) term (38 week) human placentae. (Scale bars: 50 µm). (C) Quantitative RT-PCR analyses using isolated CTBs from first trimester and term placentae and LCM-captured SynTs (mean ± SE; n = 4, $p \leq 0.001$). The plot shows more abundant PRKCI mRNA level in first trimester CTBs compared to that in term CTBs and strong repression of PRKCI mRNA expression in SynTs.

Figure 4:

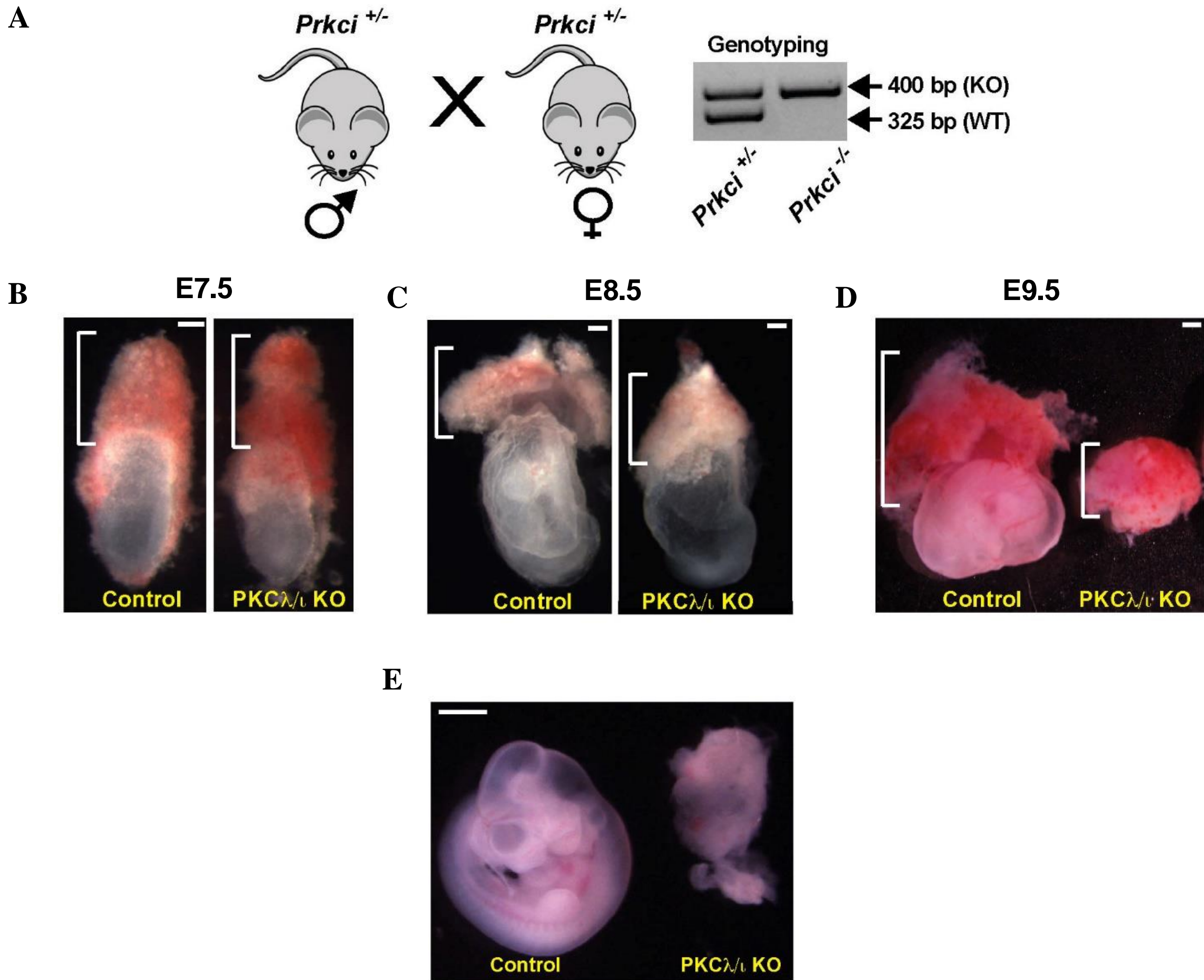


Figure 4: Global Loss of PKCλ/ι in a developing mouse embryo abrogates placentation: (A) Experimental strategy and phenotype of mouse conceptuses defining the importance of PKCλ/ι in placentation. Heterozygous *(Prkci+/-)* male and female mice were crossed to generate homozygous knockout *(Prkci-/-*, PKCλ/ι KO) embryos and confirmed by genotyping. (B), (C) and (D) Embryonic and placental developments were analyzed at E7.5, E8.5 and E9.5 and representative images are shown. At E7.5, placenta primordium developed normally in PKCλ/ι KO embryos. However, defect in placentation in PKCλ/ι KO conceptuses were observable (smaller placentae) at E8.5 and was prominent at E9.5. (Scale bars: 100 µm). (B) Developing control and PKCλ/ι KO embryos were isolated at ~E9.5 and representative images are shown. The PKCλ/ι KO embryo proper shows gastrulation defect as described in earlier studies (Scale bars: 500 µm).

Figure 5:

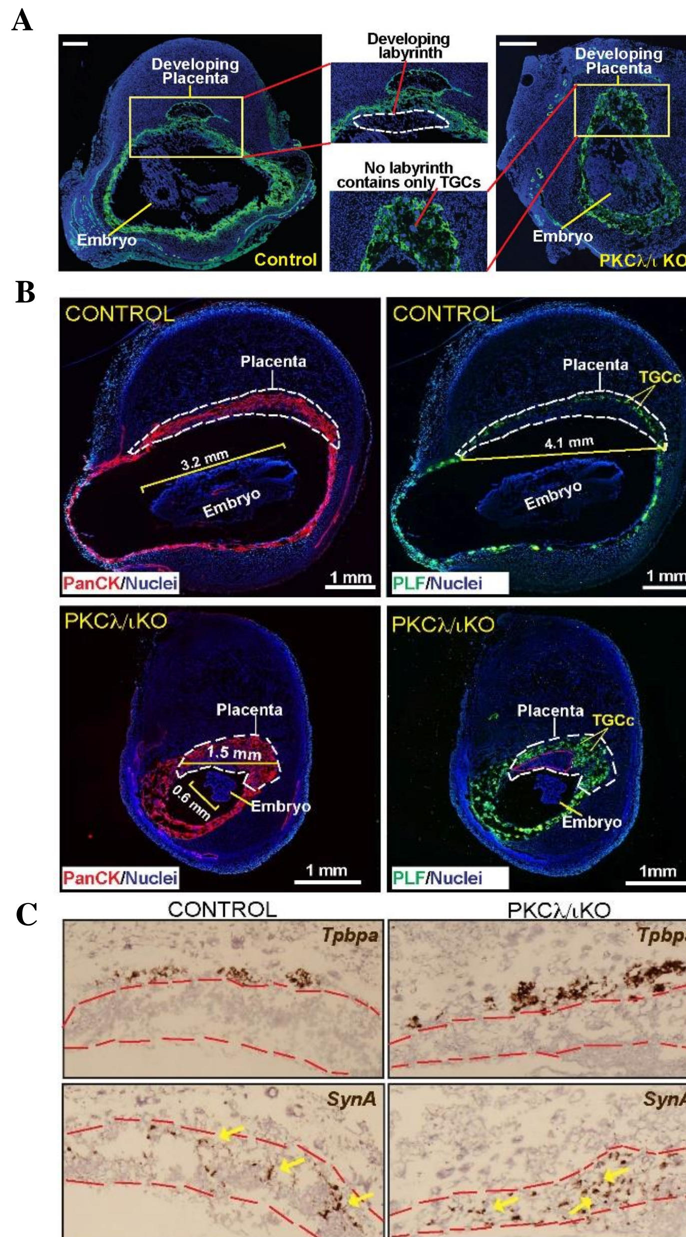


Figure 5: Global Loss of PKCλ/ι in a developing mouse embryo abrogates placentation: (A) Placentation at control and PKCλ/ι KO implantation sites were analyzed at ~E9.5 via immunostaining with anti-pan-cytokeratin antibody (green, trophoblast marker). The developing PKCλ/ι KO placenta lacks the labyrinth zone and mainly contains the TGCs (red line). Also, unlike in control embryos, the developmentally arrested PKCλ/ι KO placenta and embryo proper are not segregated and are attached together. (Scale bars: 500 μm). (B) Developing control and PKCλ/ι KO embryos were isolated at ~E9.5 and representative images are shown. The control embryo shows an organized layer of PLF-expressing (Green) parietal TGC. In contrast, the PKCλ/ι KO placenta contains multiple layers of TGCs. The PKCλ/ι KO placenta is one of the few examples, where other trophoblast cells (within red border) can be visible besides TGCs. The relative sizes of the control and PKCλ/ι KO embryo and placentae are also highlighted. (C) Control and PKCλ/ι KO placentae were analyzed via ISH to test expression of marker genes that are representative of different trophoblast cell types. The red borders indicate the developing labyrinth region. Both control and PKCλ/ι KO placentae show presence of *Tpbpa*-expressing and *SynA* expressing (yellow arrows) cells.

Figure 6:

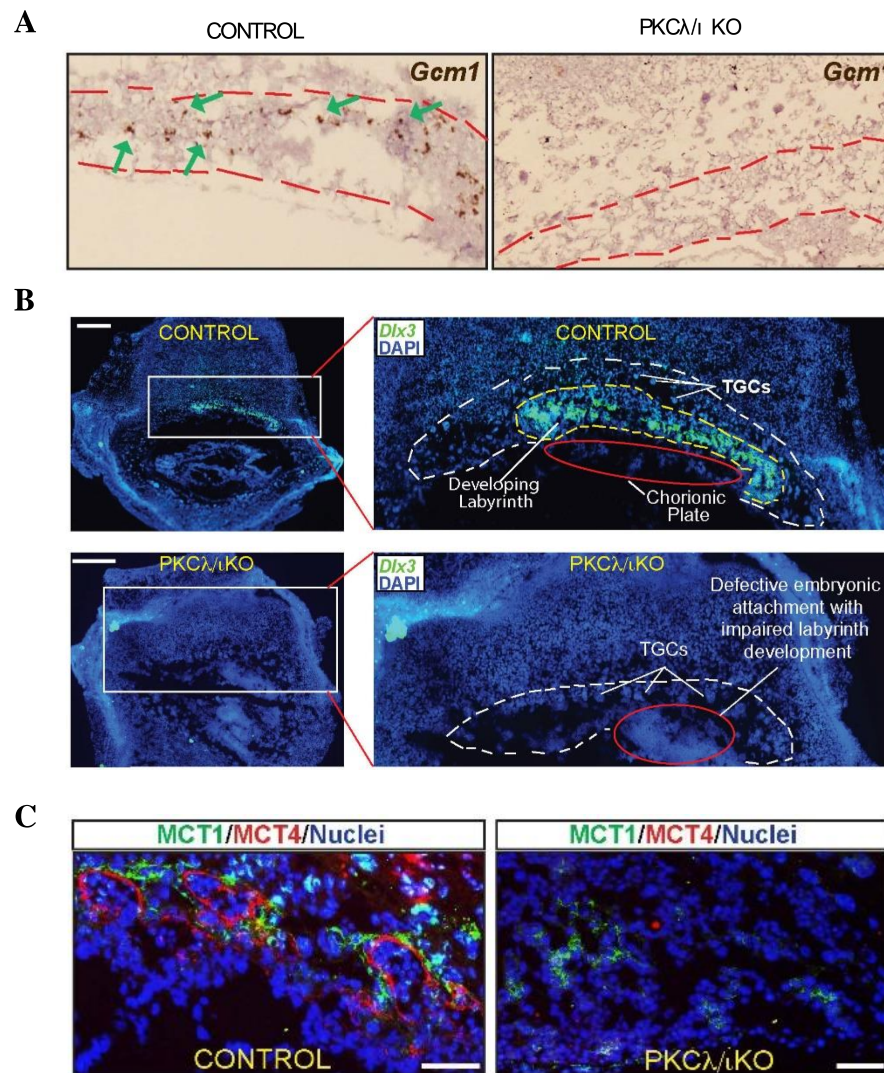


Figure 6: Global Loss of PKCλ/ι in a developing mouse embryo abrogates labyrinth morphogenesis: Control and PKCλ/ι KO placentae were analyzed via ISH to test expression of *Gcm1*-expressing cells (green arrows) were not detected in PKCλ/ι KO placenta. (B) RNA in situ hybridization assay was performed using fluorescent probes against *Dlx3* mRNA. Images show that, unlike the control placenta, the PKCλ/ι KO placenta lacks *Dlx3* expressing labyrinth trophoblast cells. (Scale bars: 500 µm). (C) Control and PKCλ/ι KO placentae were immunostained for MCT1 (green) and MCT4 (red) expression pattern to test formation of SynT-I and SynT-II populations, respectively. The fluorescence image panel for the control placenta shows formation of two continuous SynT layers. In contrast, the PKCλ/ι KO placenta lacks any MCT4-expresing cells but contains few MCT1 expressing cells. However, unlike in control embryos, the MCT1 expressing cells in PKCλ/ι KO placenta are dispersed, indicating lack of formation of a matured SynT-I population, which arise via cell-cell fusion.

Figure 7:

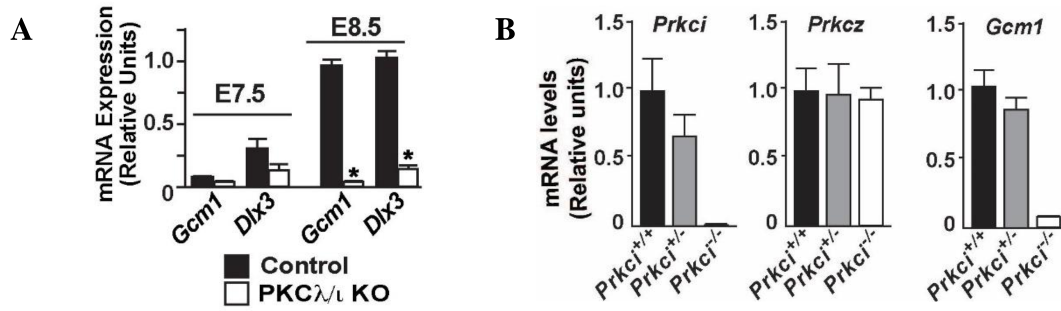


Table 1:

Analyses at E7.5

	Total No of embryos Analyzed (86) (From 12 litters)	Average length of embryo
WT	16	1 ± 0.1 mm
HET	50	1 ± 0.1 mm
KO	20	1 ± 0.1 mm

Table 2:

Analyses at E9.5

	No of embryos dissected (66 from 8 litters)	No of embryo sections analyzed (101 from 11 litters)	Average length of embryo	Average length of developing placenta with labyrinth
WT	14	29	3 ± 0.4 mm	3.1 ± 1 mm
HET	37	40	3 ± 0.4 mm	3.1 ± 1 mm
KO	15	32	0.9 ± 0.3 mm	1.5 ± 0.7mm

Figure 7: Global Loss of PKCλ/ι in a developing mouse embryo abrogates labyrinth morphogenesis: (A) Quantitative RT-PCR analyses in E7.5 and E8.5 PKCλ/ι KO placenta primordia (mean ± SE; n = 4, p≤0.001) showing abrogation of *Gcm1* and *Dlx3* induction, which happens between E7.5 and E8.5, in PKCλ/ι KO developing placentae. (B) Quantitative RT-PCR analyses using placental tissue from control and PKCλ/ι KO embryos. *Prkcz* mRNA level remained unaltered in KO placentae. PKCλ/ι KO placentae showed near complete loss of *Gcm1*. Tables 1 and 2 showing total number of embryos analyzed at E7.5 and E9.5 stages.

Figure 8:

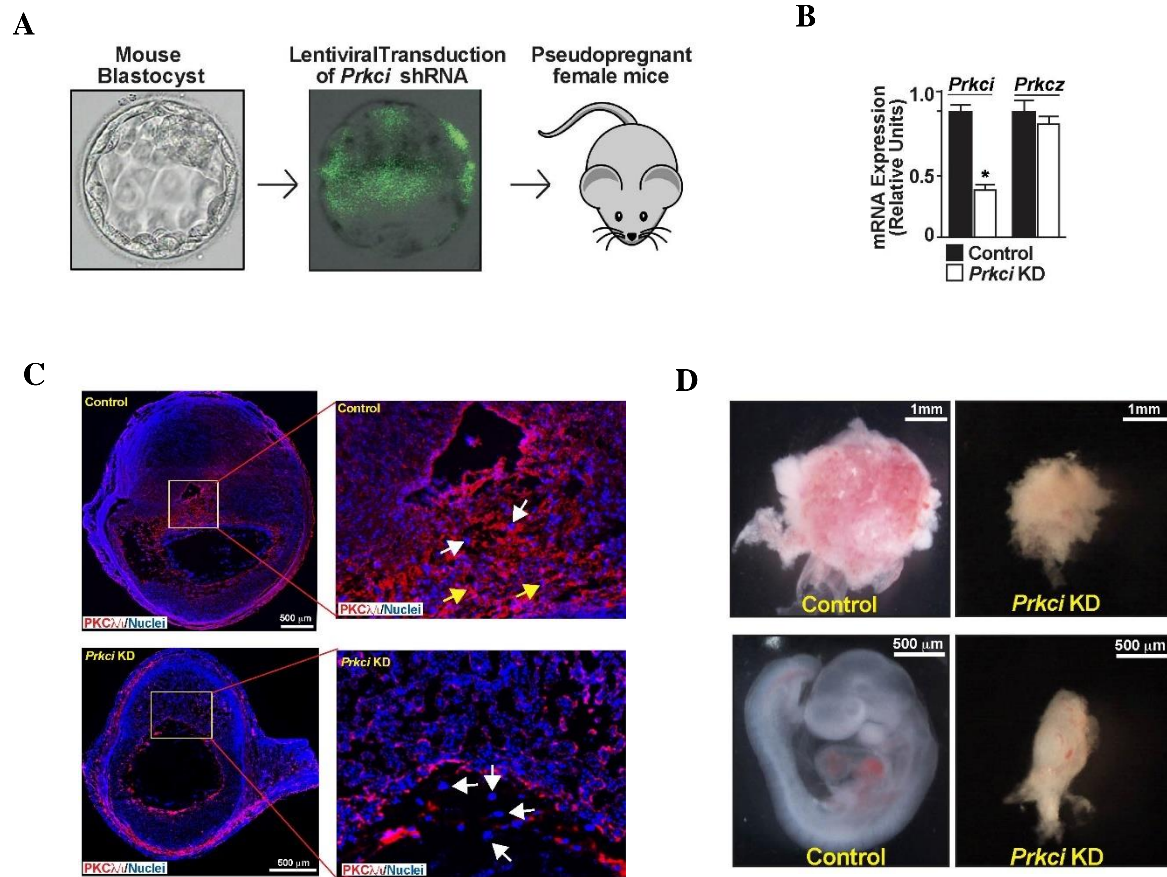


Figure 8: Trophoblast-specific PKCλ/ι depletion impairs mouse placentation leading to embryonic death: (A) Schematics to study developing mouse embryos with trophoblast-specific depletion of *Prkci* (*Prkci* KD embryos). Blastocysts were transduced with lentiviral vectors expressing shRNA against *Prkci* and transduction was confirmed by monitoring EGFP expression. Transduced blastocysts were transferred into the uterine horns of pseudo pregnant females to study subsequent effect on embryonic and placental development. (B) Knockdown efficiency with shRNA was confirmed by testing loss of *Prkci* mRNA expression in transduced blastocysts. (C) Control and *Prkci* KD mouse embryos were immunostained with anti-PKCλ/ι antibody (red) and DAPI. PKCλ/ι expression was significantly reduced in the TGCs of *Prkci* KD placenta (marked by white arrows). In contrast high level of PKCλ/ι expression was maintained in trophoblast cells of control placenta (marked by yellow arrows). (D) Representative images show control and *Prkci* KD placentae and developing embryos, isolated at E9.5. Similar to global PKCλ/ι KO embryos, trophoblast-specific *Prkci* KD embryos showed severe developmental defect.

Figure 9:

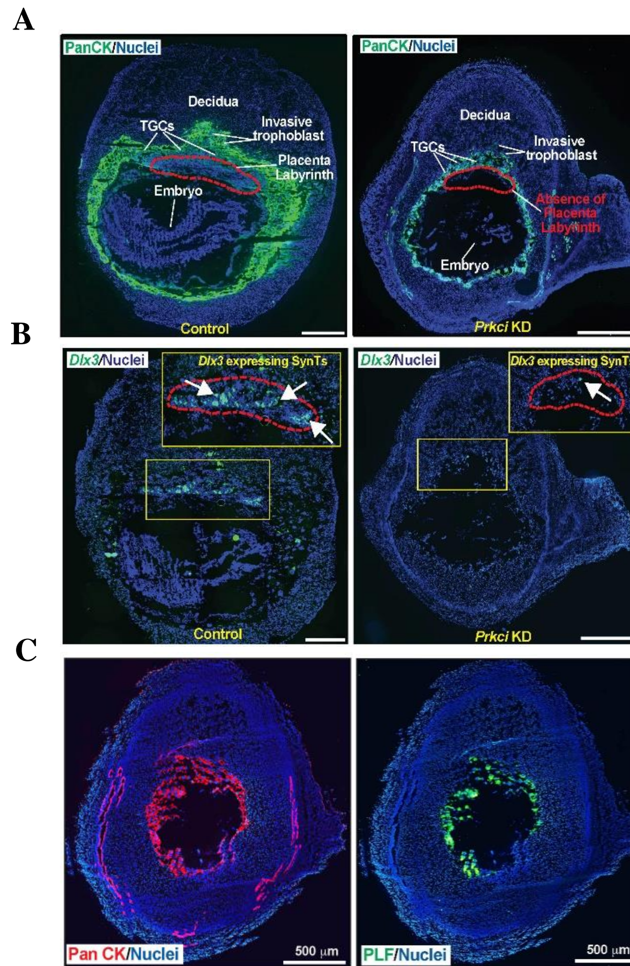


Table 3:

Total No of embryos analyzed (71 from 8 litters)	No of embryos dissected	No of embryos immunostained
Control	**18**	**17**
***Prkci* TE KD**	**21**	**15**

Figure 9: Trophoblast-specific PKCλ/ι depletion impairs mouse placentation leading to embryonic death: (A) Immunostaining with anti-pan-cytokeratin antibody (green, trophoblast marker) showed defective placentation in the *Prkci* KD implantation sites at ~E9.5. The images show that unlike the control placenta, labyrinth formation was abrogated in the *Prkci* KD placenta. (Scale bars: 500 µm). (B) RNA in situ hybridization assay confirmed near-complete absence of Dlx3 expressing trophoblast cells in the *Prkci* KD placenta. (Scale bars: 500 µm). (C) Consecutive sections of *Prkci* KD mouse embryo were immunostained with anti-Pan Cytokeratin (Pan CK, left panel, (red)] and anti-PLF (right panel, green) antibodies. Images show that almost all the Pan-CK expressing cells are PLF-expressing TGCs. Table 3 shows total number of *Prkci* KD embryos, analyzed for the study.

Figure 10:

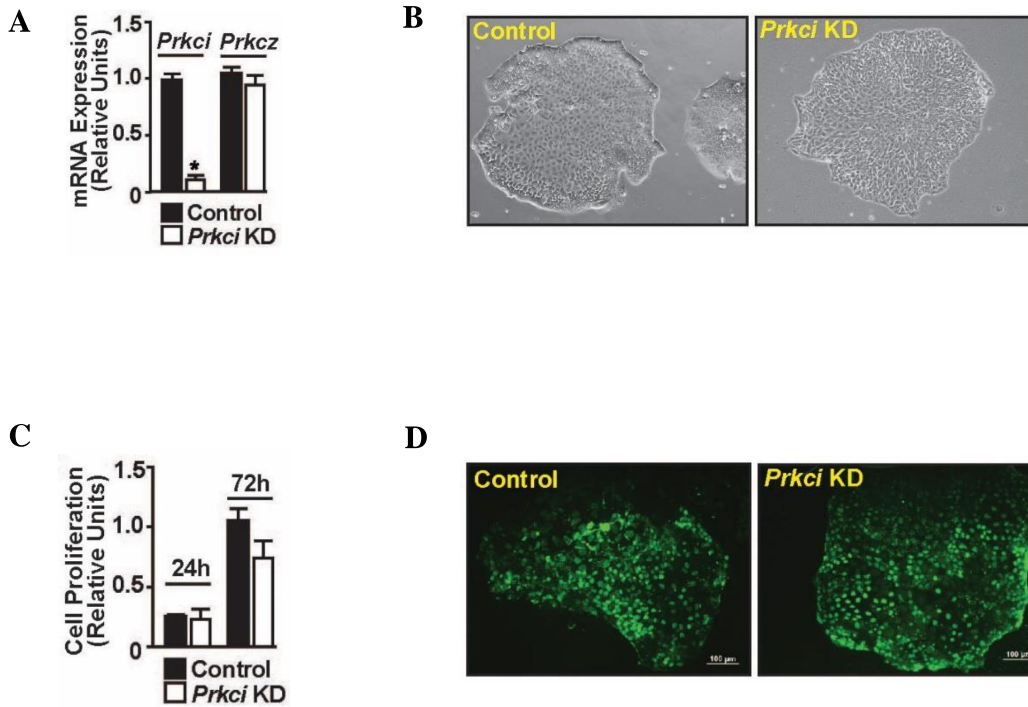


Figure 10: PKCλ/ι signaling in a developing mouse embryo is essential to establish a transcriptional program for TSPC to SynT differentiation: (A) Quantitative RT-PCR to validate loss of *Prkci* mRNA expression in *Prkci* KD mouse TSCs (mean ± SE; n = 4, $p \leq 0.001$). The shRNA molecules targeting the *Prkci* mRNA had no effect on *Prkcz* mRNA expression (B) Morphology of control and *Prkci* KD mouse TSCs. (C) and (D) Assessing proliferation rate of control and *Prkci* KD mouse TSCs by MTT assay and BrdU labeling, respectively.

Figure 11:

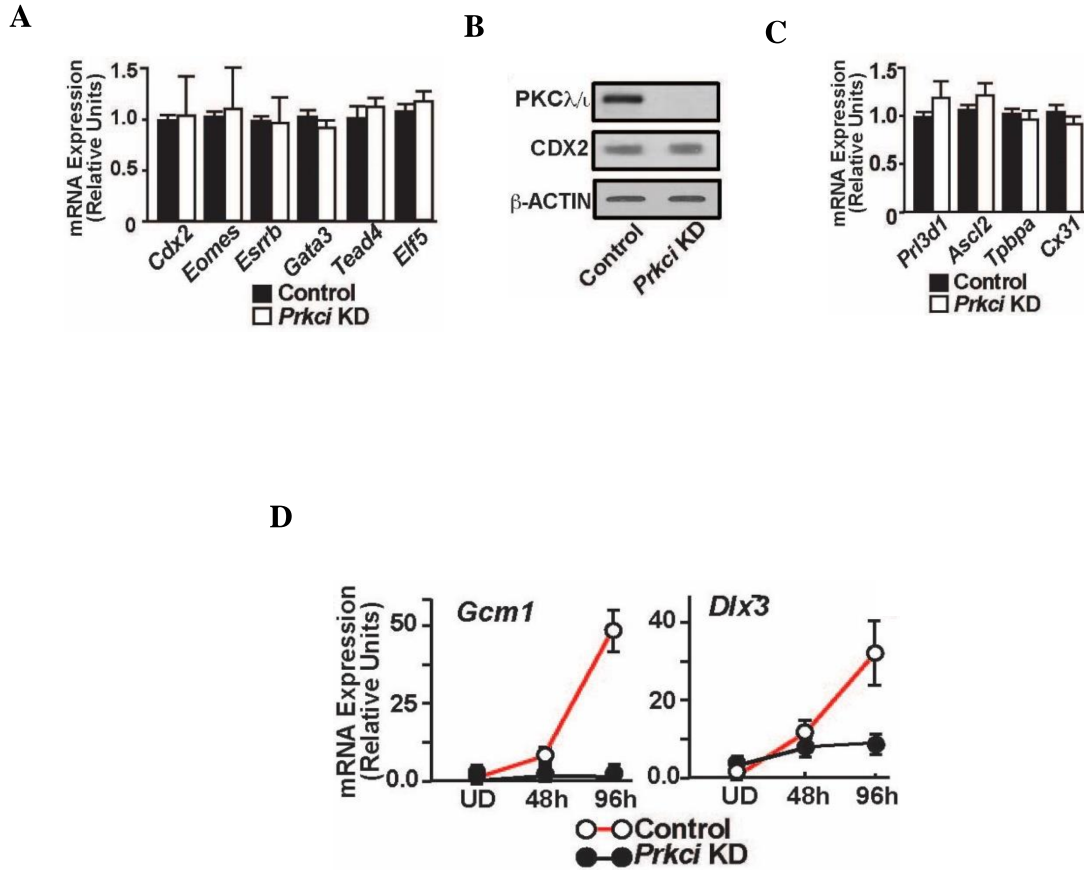


Figure 11: PKCλ/ι depletion in mouse TSPCs does not alter stem state or differentiation gene expression program: (A) Quantitative RT-PCR (mean ± SE; n = 3), showing unaltered mRNA expression of trophoblast stem-state specific genes like *Cdx2, Eomes, Tead4, Gata3, Elf5* and *Esrrb* in *Prkci* KD mouse TSCs. (B) Western blot analyses confirming unaltered CDX2 protein expression in mouse TSCs upon PKCλ/ι depletion. (C) Quantitative RT-PCR analyses of *Prl3d1, Ascl2, Tpbpa* and *Cx31* mRNA expression in differentiated *Prkci* KD mouse TSCs. (mean ± SE; n = 3) to assess TGCs, spongiotrophoblasts and glycogen trophoblast cell differentiation, respectively. Expression of these genes were not altered in differentiated *Prkci* KD mouse TSCs, indicating PKCλ/ι is dispensable for mouse TSC differentiation to TGCs, spongiotrophoblasts and glycogen trophoblast cells. (D) *Prkci* KD mouse TSCs were allowed to grow under differentiation conditions and gene expression analyses of syncytiotrophoblast markers *Gcm1* and *Dlx3* were done (mean ± SE; n = 3). The plots show that loss of PKCλ/ι in mouse TSCs results in impaired induction of *Gcm1* and *Dlx3* expression.

Figure 12:

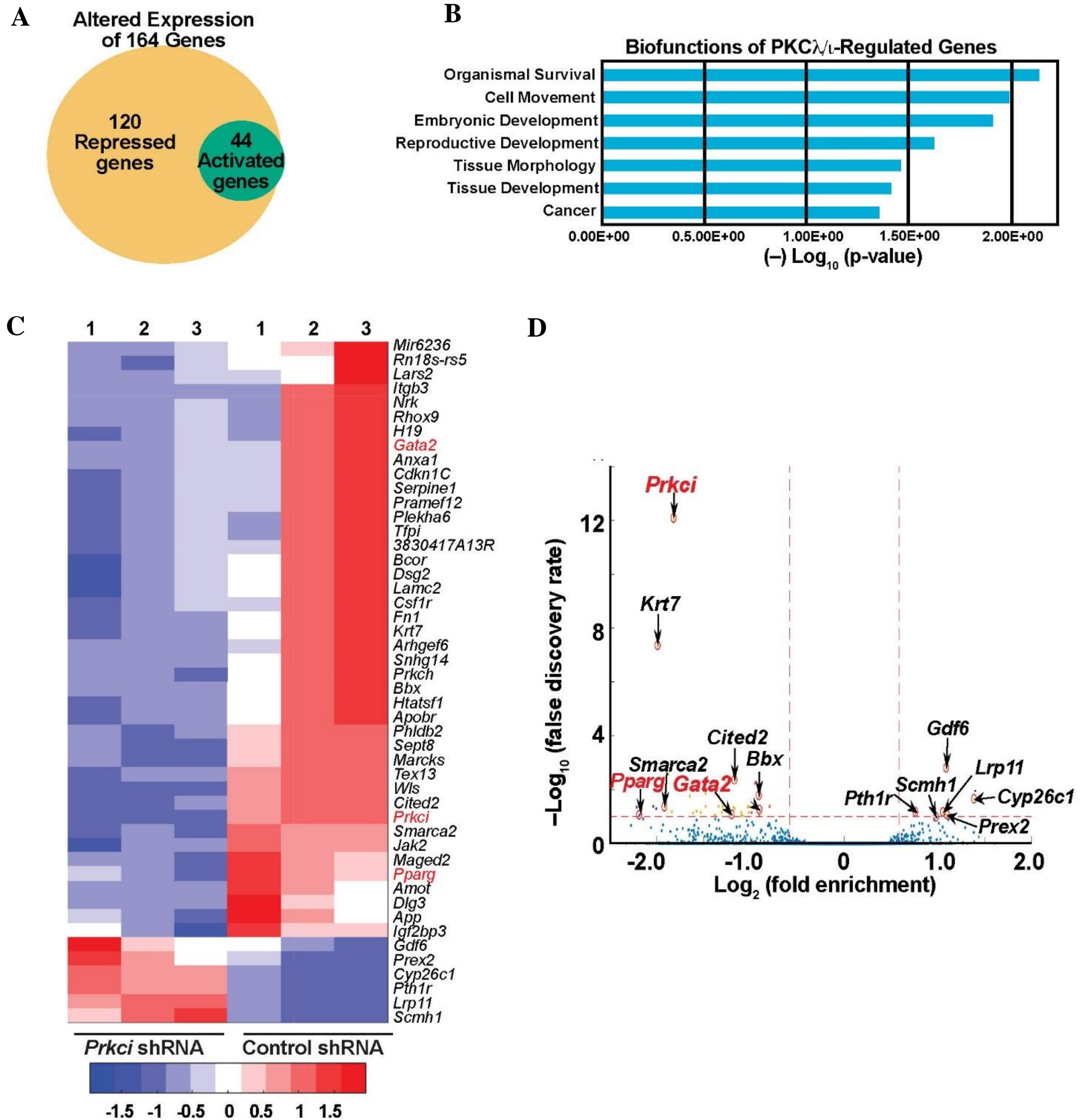


Figure 12: Downstream regulators of PKCλ/ι signaling in mouse TSPCs: (A) Whole genome RNA-seq was performed in *Prkci* KD mouse TSCs and the Venn diagram shows number of genes that were significantly downregulated (120 genes) and upregulated (44 genes) upon PKCλ/ι depletion. (B) The plot shows most significant biofunctions (identified via Ingenuity Pathway Analysis) of PKCλ/ι-regulated genes in mouse TSCs. (C) and (D) Heat map and Volcano plot, respectively, showing significantly altered genes in *Prkci* KD mouse TSCs. Along with *Prkci, Gata2* and *Pparg* (marked in red) are among the most significantly down-regulated genes in *Prkci* KD mouse TSCs.

Figure 13:

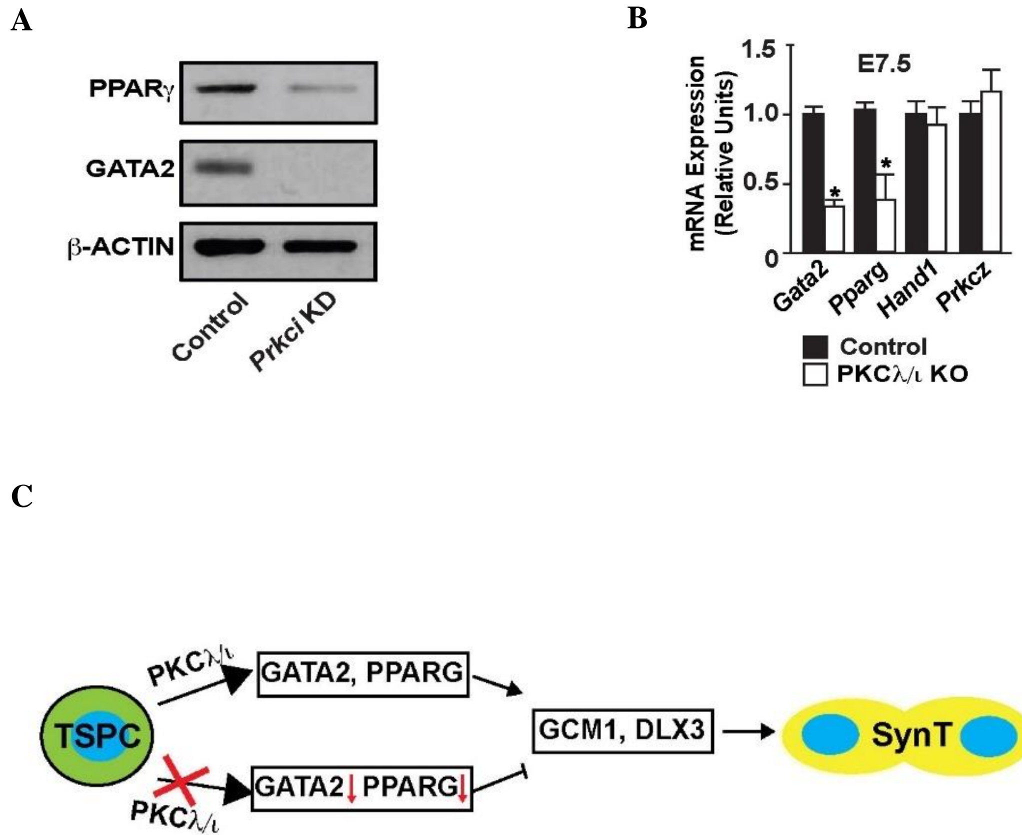


Figure 13: PKCλ/ι signaling in a developing mouse embryo is essential to establish a transcriptional program for TSPC to SynT differentiation: (E) Western blot analyses showing loss of GATA2 and PPARγ protein expressions in *Prkci* KD mouse TSCs. (F) Quantitative RT-PCR analyses showing downregulation of *Gata2* and *Pparg* mRNA expression in TSPCs of E7.5 PKCλ/ι KO placenta primordium (mean ± SE; n = 4, $p \leq 0.01$). Expression of *Hand1* and *Prkcz* mRNAs remain unaltered. (G) The model implicates a PKCλ/ι-GATA2/PPARγ-GCM1/DLX3 regulatory axis in SynT differentiation during mouse placentation.

Figure 14:

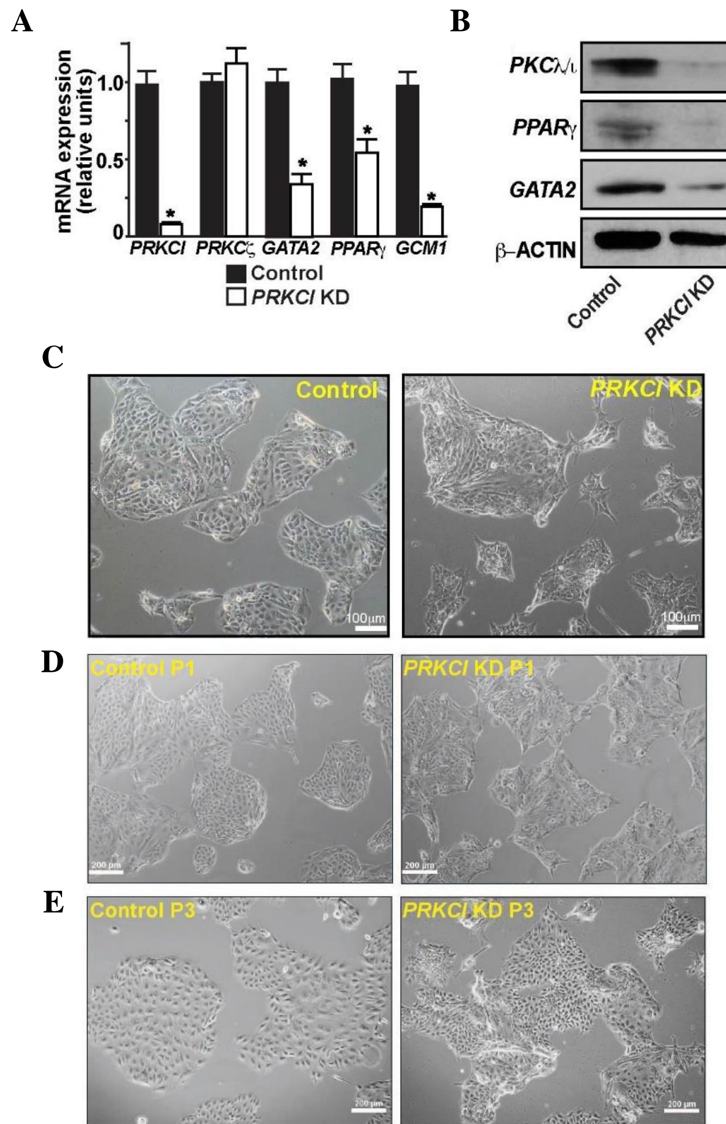


Figure 14: Downstream regulators of PKCλ/ι signaling is conserved between human and mouse TSCs: (A) Quantitative RT-PCR analyses showing loss of *GATA2, PPARG* and *GCM1* mRNA expression (mean ± SE; n = 4, $p \leq 0.01$) upon knock down of *PRKCI* in human TSCs (PRKCI KD human TSCs). The mRNA expression of *PRKCZ* was not affected. (B) Western blots show loss of PKCλ/ι, GATA2 and PPARγ protein expressions in PRKCI KD human TSCs. (C) Micrographs showing morphology of control and *PRKCI* KD human TSCs. (D) and (E) Micrographs show maintenance of stem state colony morphology of both control and *PRKCI* KD human TSCs for multiple passages.

Figure 15:

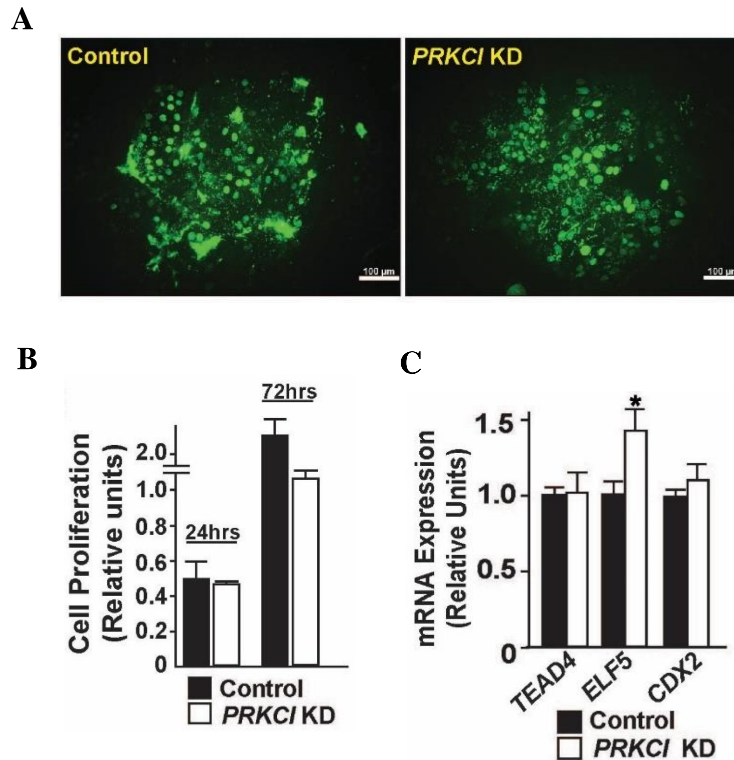


Figure 15: PKCλ/ι depletion in human TSCs does not alter stem state gene expression program: (A) Immunofluorescence images show incorporation of BrdU in both control and *PRKCI* KD human TSCs, indicating maintenance of cell proliferation. (B) Quantitative assessment of cell proliferation rate of control and *PRKCI* KD human TSCs by MTT assay. (C) Quantitative RT-PCR analyses (mean ± SE; n = 4, $p \leq 0.01$) showing relative mRNA expression of trophoblast stem state genes in control and *PRKCI* KD human TSCS.

Figure 16:

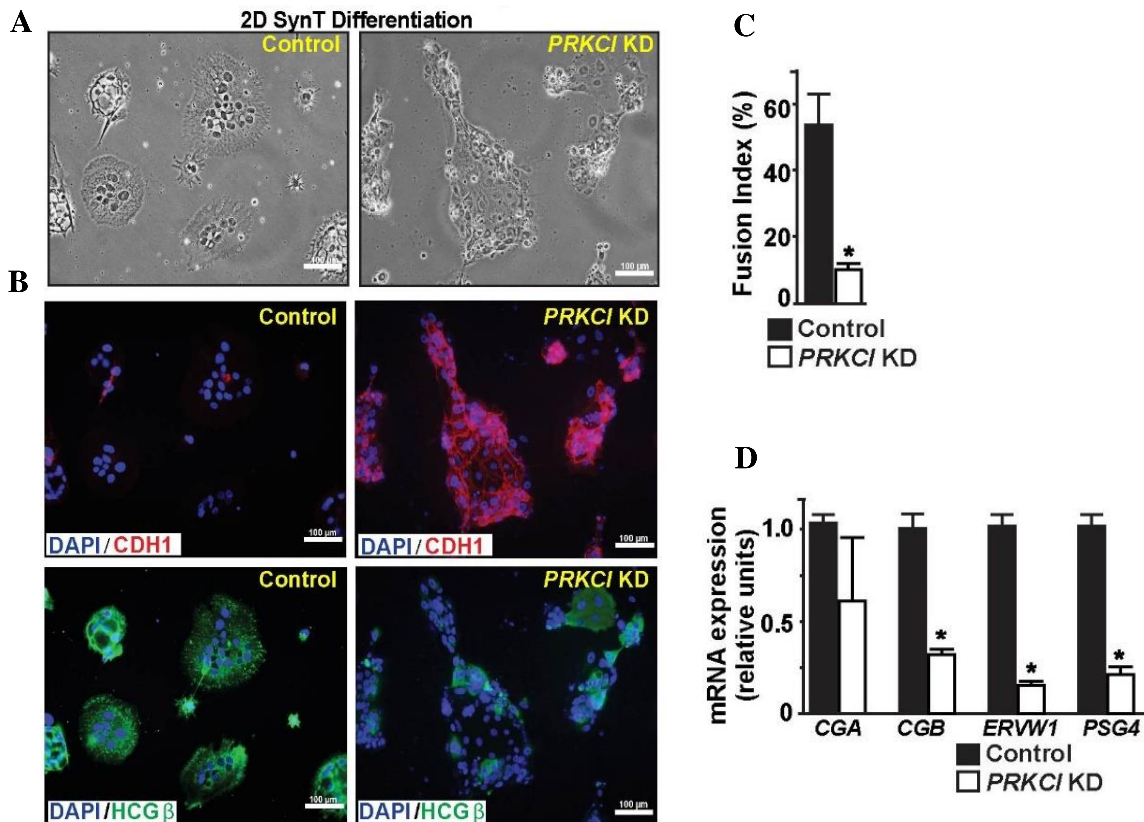


Figure 16: Loss of PKCλ/ι impairs SynT differentiation potential in human TSCs: (A) Control and *PRKCI* KD human TSCs were subjected for 2-dimensional (2D) SynT differentiation on collagen coated adherent cell culture dishes. Image panels show altered cellular morphology (B) maintenance of E-Cadherin (CDH1) expression (Upper right panel in B); and impaired induction of HCGβ expression (lower right panel in B) in *PRKCI* KD human TSCs. (C) Cell fusion index was quantitated in control and *PRKCI* KD human TSCs. Fusion index was determined by measuring number of fused nuclei with respect to total number of nuclei within image fields (5 randomly selected fields from individual experiments were analyzed, 3 independent experiments were performed) (D) Quantitative RT-PCR analyses showing significant (mean ± SE; n = 4, $p \leq 0.001$) downregulation of mRNA expressions of SynT-specific markers in *PRKCI* KD human TSCs, undergoing 2D SynT differentiation.

Figure 17:

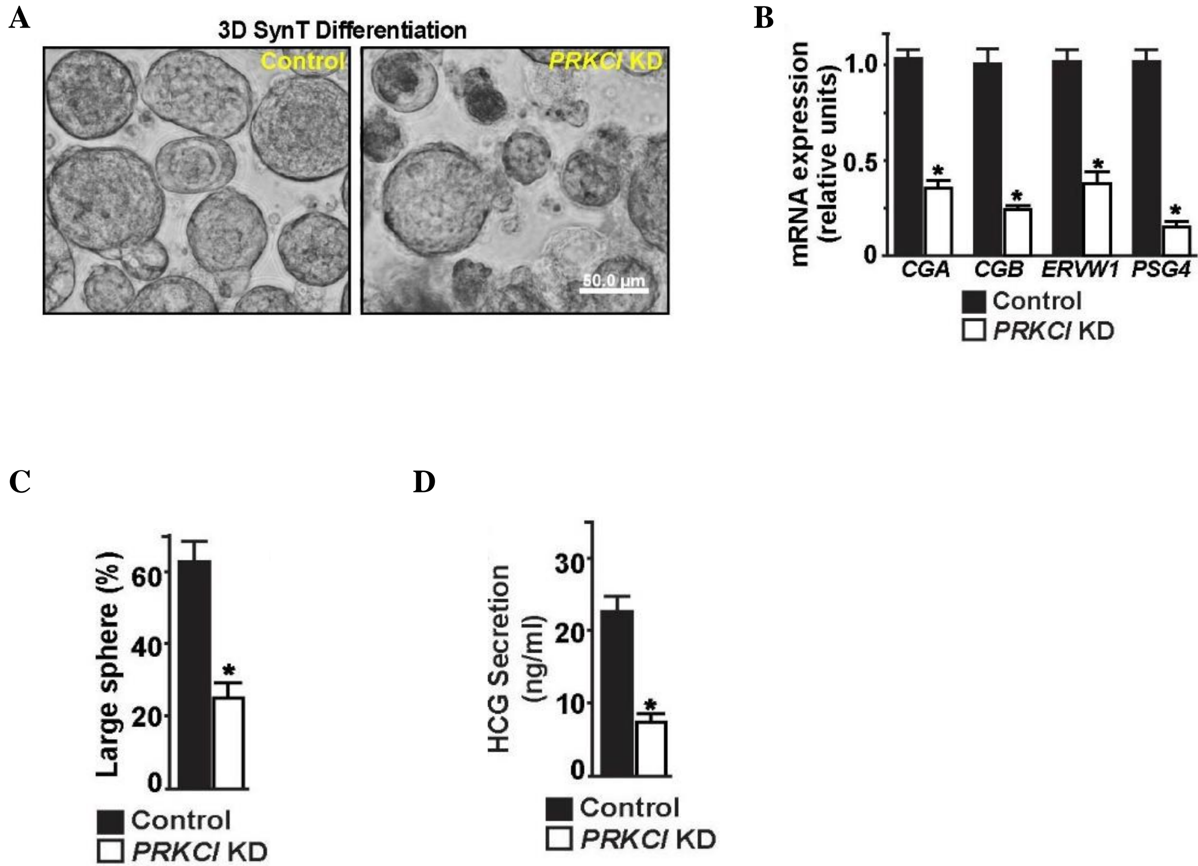


Figure 17: Loss of PKCλ/ι impairs SynT differentiation potential in human TSCs: (A) Control and *PRKCI* KD human TSCs were subjected to 3-dimensional (3D) SynT differentiation on low attachment dishes. Micrographs show defective SynT differentiation, as assessed from formation of large cell-spheres, in *PRKCI* KD human TSCs. (B) Quantitative RT-PCR analyses (mean ± SE; $n = 4$, $p \leq 0.001$) reveal impaired induction of SynT markers in PRKCI KD human TSCs, undergoing 3D SynT differentiation. (C) Quantification of 3D SynT differentiation efficiency was done by counting large cell-spheres (>50µm) from multiple fields (3 fields from each experiment, 3 individual experiments). (D) The plot shows relative levels of HCG, measured by ELISA with culture medium (mean ± SE; $n = 3$, $p \leq 0.001$) from control and *PRKCI* KD human TSCs, undergoing SynT differentiation.

Figure 18:

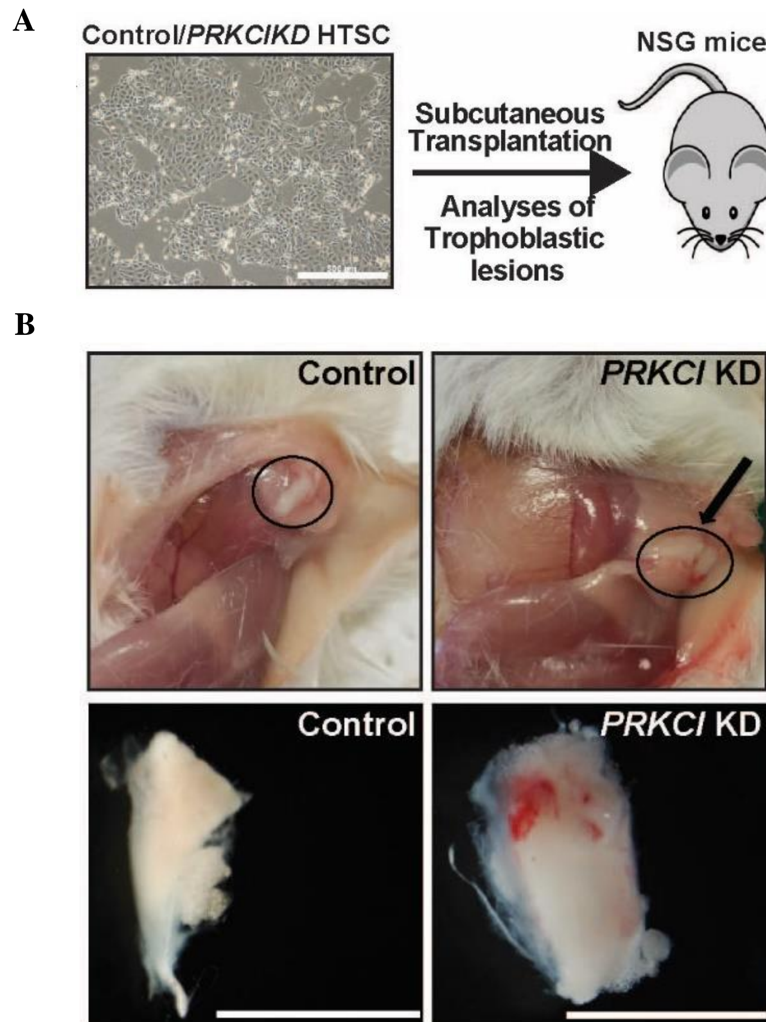


Figure 18: PKCλ/ι depleted human TSCs form trophoblastic lesions upon transplantation in NSG mice: (A) Schematics of testing in vivo developmental potential of human TSC via transplantation assay in NSG mice. Control and *PRKCI* KD human TSCs were mixed with matrigel and were subcutaneously injected into the flank of NSG mice. (B) Images show trophoblastic lesions that were developed from transplanted human TSCs. (Scale bars: 1 cm).

Figure 19:

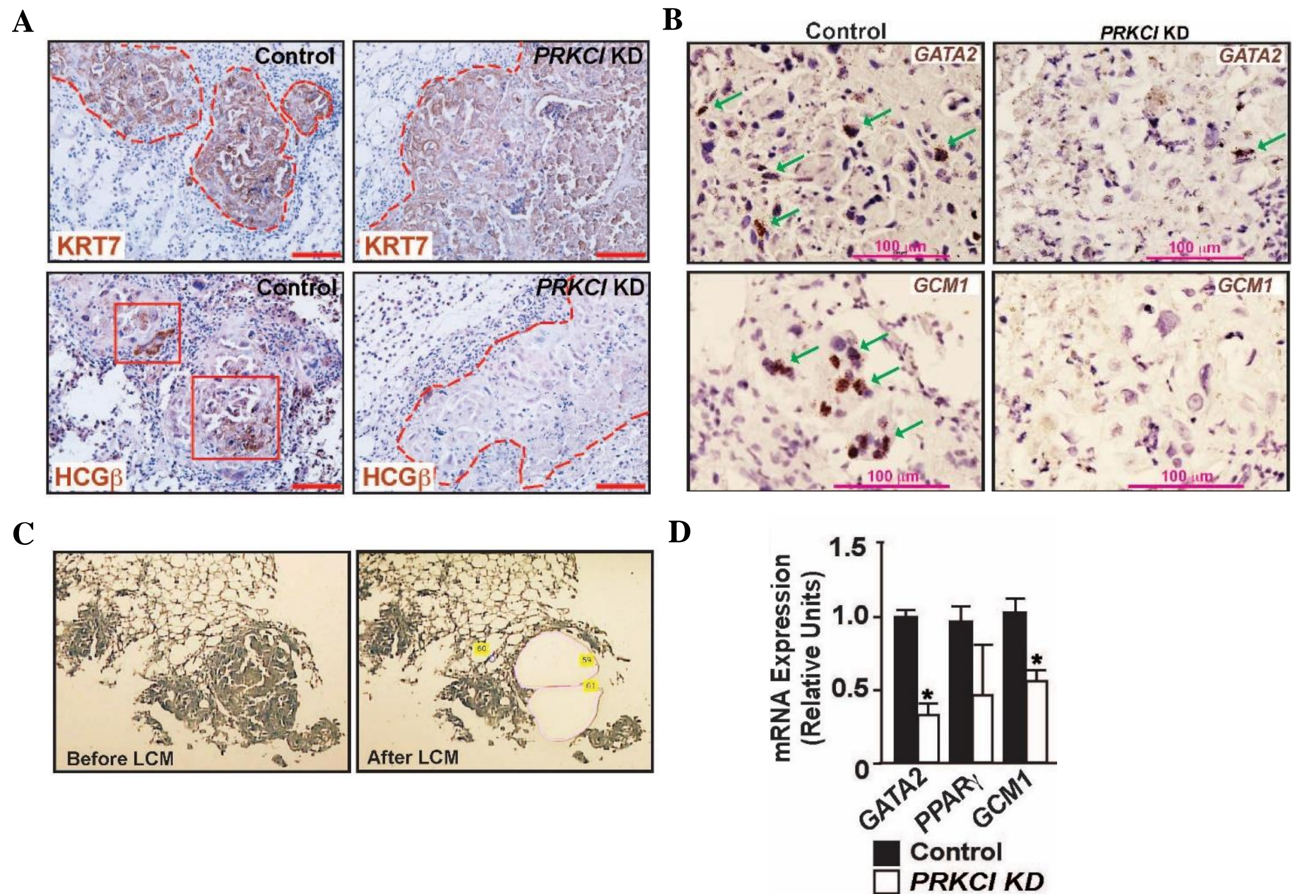


Figure19: PKCλ/ι signaling is essential for in vivo SynT differentiation potential of Human TSCs: (A) Trophoblastic lesions were immunostained with anti-human cytokeratin7 (KRT7) antibody and anti-human HCGβ antibody, respectively. The trophoblastic lesions that were developed from both control and *PRKCI* KD human TSCs contained KRT7 expressing human trophoblast cells but the lesions from *PRKCI* KD human TSCs lacked HCGβ expressing trophoblast populations (lower right panel), indicating impairment in SynT developmental potential. (Scale bars: 500 μm). (B) The reduced levels of *GATA2* and *GCM1* mRNA expression were confirmed by ISH. (C) Regions of trophoblastic lesion obtained after transplantation of control and *PRKCI* KD human TSCs in immunocompromised mice were captured via LCM for quantitative mRNA expression analyses. Images show example of such a region before and after cell capturing. (D) Quantitative RT-PCR analyses using LCM-captured cell from trophoblastic lesions (mean ± SE; n = 3, $p \leq 0.01$). The plot shows significant downregulation of both *GATA2* and *GCM1* mRNA expressions in lesions, derived from *PRKCI* KD human TSCs. The *PPARG* mRNA level was also reduced but the data was not statistically significant.

DISCUSSIONS

In this study, using both mouse knockout models and human TSCs we have uncovered an evolutionary conserved function of PKCλ/ι signaling during trophoblast development and mammalian placentation. In recent years, placental development and the maternal-fetal interaction is being studied with considerable interest as defects in placentation in early post-implantation embryos can lead to either pregnancy failure (K. Cockburn & J. Rossant, 2010; Pfeffer & Pearton, 2012; R. M. Roberts & S. J. Fisher, 2011; J. Rossant & J. C. Cross, 2001), or pregnancy-associated complications like Intrauterine Growth Restriction (IUGR) and preeclampsia (Myatt, 2006; Pfeffer & Pearton, 2012; Redman & Sargent, 2005; J. Rossant & J. C. Cross, 2001), or serves as developmental causes for postnatal or adult diseases (Funai et al., 2005; Gluckman et al., 2008; Godfrey & Barker, 2000). Establishment of the intricate maternal-fetal relationship is instigated by development of the SynT populations, which not only establish the maternal-fetal exchange surface but also modulate the immune function and molecular signaling at the maternal-fetal interface to assure successful pregnancy (Aplin et al., 2017; Pavlicev et al., 2017). Our findings in this study indicate that PKCλ/ι-regulated optimization of gene expression is fundamental to SynT development.

One of the interesting findings of this study is the specific requirement of PKCλ/ι in establishing the SynT differentiation program. The lack of PKCλ/ι expression in SynTs within first trimester and term human placentae indicates that PKCλ/ι signaling is not required for maintenance of SynT function. The specific need of PKCλ/ι to "prime" trophoblast progenitors for SynT differentiation is further evident from the phenotype of PKCλ/ι-null mouse embryos. Although PKCλ/ι is expressed in pre-implantation embryos and in TSPCs of placenta primordium, it is not essential for trophoblast cell lineage development at those early stages of embryonic development. Also, the development of TGCs in PKCλ/ι-null placentae indicates that PKCλ/ι is dispensable for

the development of TGCs, a cell type that is equivalent to human EVTs. Rather, PKCλ/ι is essential for labyrinth development at the onset of chorio-allantoic attachment, indicating a specific need of PKCλ/ι in nascent SynT population.

Earlier studies with gene knockout mice indicated that GCM1 is essential for the placental labyrinth development. The *Gcm1* knockout mice die at E10.5 (Schreiber, Riethmacher-Sonnenberg, Riethmacher, Tuerk, Enderich, Bosl, et al., 2000) with major defect in SynT development. Our findings in this study show that the PKCλ/ι signaling is essential to induce both *Gcm1* expression and development of the SynT-II population. We detected presence of a SynA/MCT1-expressing, putative SynT-I trophoblast population in a few PKCλ/ι-KO placentae. However, development of a matured SynT-I layer was impaired in all PKCλ/ι-KO placentae.

During mouse placentation, *Dlx3* is initially induced in basal EPC progenitors, which constitutes a layer in chorion and eventually differentiate to SynT-I lineage. Later, *Dlx3* is broadly expressed in both SynT-I and SynT-II population. Defect in SynT development and placental labyrinth formation is also evident in the *Dlx3* knockout mice by E9.5 (Morasso, Grinberg, Robinson, Sargent, & Mahon, 1999). We found that both *Gcm1 and Dlx3* transcriptions are induced between E7.5-E8.5, a developmental stage when labyrinth development is initiated upon chorio-allantoic attachment and this induction is impaired in PKCλ/ι-KO placentae. Not surprisingly, the PKCλ/ι-KO embryos show a more severe phenotype with complete absence of matured SynTs in the developing placentae.

Our unbiased gene expression analyses in *Prkci* KD mouse TSCs strongly indicate that the PKCλ/ι signaling ensures TSPC to SynT transition by maintaining expression of two conserved transcription factors, GATA2 and PPARγ. Both GATA2 and PPARγ are known regulators of SynT

development. We showed that *Gcm1* and *Dlx3* are direct target genes of GATA2 in mouse TSCs (P. Home et al., 2017). Also loss of PPARγ in mouse TSCs is associated with complete loss of *Gcm1* induction during TSC to SynT differentiation (Mana M. Parast et al., 2009). Thus, the impairment of *Gcm1* and *Dlx3* induction upon loss of PKCλ/ι-KO during TSPCs to SynT transition could be a direct result of the downregulation of GATA2 and PPARγ.

In this study, we also found that PKCλ/ι-mediated regulation of GATA2, PPARγ and GCM1 is a conserved event in the mouse and human TSCs. In a recent study (Milano-Foster et al., 2019), we have shown that GATA2 regulates human SynT differentiation by directly regulating transcription of key SynT-associated genes, such as *CGA*, *CGB*, and *ERVW1*, via formation of a multi-protein complex, including histone demethylase KDM1A and RNA polymerase II, at their gene loci. Based on the conserved nature of GATA2 and PPARγ expression and their regulation by PKCλ/ι in both mouse and human TSCs, we propose that a conserved PKCλ/ι-GATA2/PPARγ-GCM1 regulatory axis instigates SynT differentiation during mammalian placentation. We also propose that the PKCλ/ι-GATA2/PPARγ signaling axis mediates the progenitor to SynT differentiation by modulating global transcriptional program, which also involves epigenetic regulators, like KDM1A. As small molecules could modulate function of most of the members of this regulatory axis, it will be intriguing to test whether or not targeting this regulatory axis could be an option to attenuate SynT differentiation during mammalian placentation.

Another interesting finding of our study is impaired placental and embryonic development upon loss of PKCλ/ι expression specifically in trophoblast cell lineage. The trophoblast-specific PKCλ/ι depletion largely recapitulated the placental and embryonic phenotype of the global PKCλ/ι-knockout mice. These results along with selective abundance of the PKCλ/ι protein expression in TSPCs of post-implantation mouse embryos indicated that the defective embryo

patterning in global PKCλ/ι-KO mice is probably an effect of impaired placentation in those embryos. It is well known that the cells of a gastrulating embryo have signaling cross talks with the TSPCs of a placenta primordium. Furthermore, defect in placentation is a common phenotype in many embryonic lethal mouse mutants. However, we have a poor understanding about how an early defect in labyrinth formation leads to impairment in embryo patterning and the specific phenotype of PKCλ/ι-knockout mice provides an opportunity to better understand this process. Unfortunately, we do not have access to PKCλ/ι-conditional knockout mouse model and the lentiviral-mediated shRNA delivery approach did not provide us with an option to deplete PKCλ/ι in specific trophoblast cell types. During the completion of this study, we are also establishing a new mouse model, in which we will be able to conditionally delete PKCλ/ι in specific trophoblast cell types to better understand how PKCλ/ι functions in nascent SynT lineage and how it regulates the cross talk between the developing embryo proper and the developing placenta. Also, our finding in this study is the first known implication of PKCλ/ι-signaling in human trophoblast lineage development and function. As defective SynT development could be associated with early pregnancy loss or pregnancy-associated disorders including fetal growth restriction, we also plan to study whether defective PKCλ/ι function or associated downstream mechanisms are associated with early pregnancy loss and pregnancy associated disorders.

Chapter 3: Loss of PKCλ/ι signaling in Gcm1 expressing syncytiotrophoblasts causes embryonic lethality

INTRODUCTION

The maternal-fetal interface in the murine placenta is made up of the tri-laminar trophoblast, where sinusoidal trophoblast giant cell (S-TGC) and two consecutive syncytiotrophoblast cell layers (SynT-I and SynT-II) separate maternal blood sinusoids from fetal vasculature. The S-TGCs are loosely connected to the underlying SynT layers by desmosomes allowing these cells to access maternal blood directly. The syncytial layers arise from different trophoblast populations. With the SynT-I, progenitors arising in the ectoplacental cone after collapse of the EPC cavity whereas cells of the SynT-II arise from the basal chorionic trophoblasts. The two syncytial layers are connected by tight junctions as observed by electron microscope (David G. Simmons et al., 2008). Histological examinations of the murine labyrinth have revealed markers demarcating each of the cell layers specifically. *Cebpa, Synb* were first detected at E8.5 in certain cell clusters of the chorio-allantoic interface and in the cells at the point of invagination or initiation of folding of the fetal mesothelium. However, *Dlx3, Nr6a1* and *Cebpb* were detected as early as E7.5 in certain cell clusters of the ectoplacental cone, at the basal chorionic trophoblasts at E8.5 and more broadly throughout the chorion in later stages. *Syna* was detected as early as E8.5 in the apical cells of chorion and also in later stages in close proximity to maternal blood sinusoids but not in S-TGCs. Syna/ Synb specifically demarcates SynT-I and SynT-II respectively with Gcm1 being a SynT-II restricted marker.

Glial cells missing 1 (Gcm1) was first identified in Drosophila where the gene coding this transcription factor with a DNA binding GCM domain, was found to be essential for neuronal differentiation (Hosoya, Takizawa, Nitta, & Hotta, 1995; Jones, Fetter, Tear, & Goodman, 1995). Cells missing *Gcm* gene underwent differentiation only towards neuron. However, cells where Gcm1 is ectopically expressed formed excess of glial cells instead of neuronal differentiation.

However, it was analyzed and found that there were only two murine *gcm1* gene indicating very few members and a small family of this transcription factor, exists in mammals. RNA *in situ* hybridization analyses reveals that *Gcm1* mRNA is expressed as early as E7.5 in a subset of trophoblast cells in the chorion. *Gcm1* expression is more pronounced in specific 3-6 expressing cells separated by 4-6 *Gcm1* non-expressing cells at E8.5 at the chorio-allantoic interface. These *Gcm1* expressing cells mark the points of invagination during labyrinth branching morphogenesis at E8.5. By E9.5, *Gcm1* mRNA is more widely expressed throughout the labyrinth. Gcm1 expression decreases in the labyrinth after E17.5, indicating more of a dynamic expression pattern in murine placenta. However, Gcm1 expression was not detected in the other trophoblast cell types of the ectoplacental cone or in the trophoblast giant cells (Basyuk et al., 1999a).

Gcm1 mutant mice die in utero by E10 due to failure to undergo labyrinth branching morphogenesis at the chorio-allantoic interface leading to placental failure and embryonic lethality (Anson-Cartwright et al., 2000a) . In these mutant mice, the chorionic plate remains 'flat' and fails to interdigitate in order to form villous of the labyrinth. The chorionic trophoblast cells of the Gcm1 mutants failed to undergo fusion to give rise to syncytiotrophoblasts indicating placental defects. Gcm1 is also expressed in human cytotrophoblasts of the chorionic villi indicating a conserved functional importance of Gcm1 in the placental vasculature development.

Our previous studies indicate the functional importance of a Protein Kinase C isoform, Atypical PKCλ/ι in syncytiotrophoblast development. Our previous analysis using global *Prkci* knockout mouse model indicates a trophoblast specific PKCλ/ι dependent molecular mechanism essential for labyrinth morphogenesis. Loss of PKCλ/ι signaling leads to impaired differentiation towards SynTs leading to fetal demise at around E9.5. Trophoblast giant cells (TGC) as well as spongiotrophoblast remain unaffected upon PKCλ/ι deletion. Also, trophoblast specific depletion

of PKCλ/ι causes arrested embryonic development and recapitulates defective placental labyrinth phenotype. Our results indicate the essentiality of PKCλ/ι in the induction of differentiation of trophoblast stem and progenitor cells (TSPCs) towards syncytiotrophoblast development. We also found PKCλ/ι dependent conserved signaling ensures the establishment of human syncytiotrophoblast from cytotrophoblast progenitors using human trophoblast stem cells. Our analyses using mouse trophoblast stem cells also attested to the abovementioned results. Whole genome sequencing analyses revealed that Gata2 and PPARG (both essential for syncytiotrophoblast development) as downstream regulators of PKCλ/ι signaling in TSPCs. *Prkci* mutant embryos lacked *Dlx3* expression in the chorionic plate and *Gcm1* expressing SynT-II cells. However, there was no effect of the *Tpbpa* expressing spongiotrophoblasts or proliferin expressing trophoblast giant cells attesting to the fact that PKCλ/ι signaling is specifically essential for labyrinth development.

In this section, we have used a conditional *Prkci* knockout mouse model and our results show that *Prkci* deletion specifically in the *Gcm1* expressing cells causes embryonic lethality. We have used cre-mediated deletion of loxp site flanking exon 5 of *Prkci* gene, which resulted in the generation of a truncated protein. Our preliminary results so far indicate that loss of PKCλ/ι signaling in Gcm1 expressing cells of the labyrinth causes placental failure followed by fetal demise.

MATERIALS AND METHODS

To generate conditional knockout mouse model, exon 5 was targeted for excision as it is located towards the N terminal part of the protein and deleting this exon, causes frameshift mutation leading to synthesis of a truncated non-functional protein. LoxP sites were targeted as shown in the map below. EcoRI restriction sites were inserted within the LoxP sites of the 5' and the 3' in order to detect the size difference by PCR genotyping and RFLP.

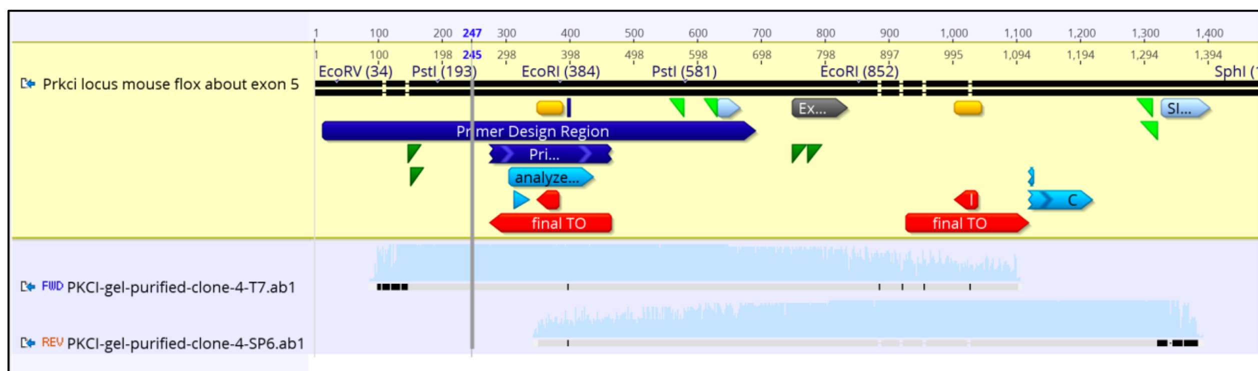


Figure 1: Cloning strategy for targeting exon 5 of Prkci gene locus

Genotyping primers:

Gene	Forward	Reverse
Prkci 3'	*TTTCCAAACAGTGAGATTAAAGGGC*	*GCCGGTGGAGAAAGCTGTATT*
Prkci 5'	*CAGTCAGTGAGTAGTACCGCTT*	*GGAGCGAGGGCTTTGATAATG*

Collection and analyses of mouse embryos: *Prkcifl/fl; Gcm1 cre* (Males) were crossed with *Prkcifl/fl* (Females) and embryos were analyzed at different gestational days. Pregnant female animals were identified by presence of vaginal plug (gestational day 0.5) and embryos were harvested at various gestational days. Uterine horns from pregnant females were dissected out and

individual embryos were analyzed under microscope. Tissues for histological analysis were kept in dry-ice cooled heptane and stored at -80 for cryo-sectioning. Yolk sacs from each of the dissected embryos were collected and genomic DNA preparation was done using GenElute mammalian Genomic DNA Miniprep Kits (Sigma- G1N70). PCR was performed using the abovementioned primers followed by EcoRI restriction digestion of the PCR products. The digested products were analyzed by agarose gel electrophoresis.

RESULTS

PKCλ/ι expression in Gcm1 expressing SynT-II cells is essential for embryonic development:

In earlier studies using global knockout mouse model, we established the importance of PKCλ/ι signaling in labyrinth morphogenesis. We found that in PKCλ/ι depleted placenta, there was complete absence of Gcm1 expressing SynT-II cells, however we could observe some dispersed SynT-I cells which failed to generate the intricate labyrinthine structure of the mouse placenta. These observations led us to interrogate further and develop a *Prkci* conditional knockout mouse model where we can deplete PKCλ/ι signaling in specific trophoblast cell types. Our primary focus was to deplete PKCλ/ι in the Gcm1 expressing SynT-II population. In order to achieve that, we generated founder animals having LoxP sites flanking exon 5 of *Prkci* gene. In the presence of cre (cyclization recombinase) enzyme, loxP sites will undergo recombination leading to excision of the exon. This excision results in the generation of a truncated protein readily identified and degraded within the cells expressing the cre enzyme.

Since the *Gcm1* cre is only expressed from the male germline and is functionally active, we set up matings where the male animal has the genotype *Prkcif/+; Gcm1 cre* and the female animal is *Prkci fl/fl*. We analyzed across many generations of litters and found that homozygous *Prkci fl/fl; Gcm1 cre* is embryonic lethal and dies in utero. Table 4 shows the total number of litters analyzed. We have analyzed 34 pups from 8 litters obtained from different mating parents.

Loss of PKCλ/ι expression in SynT-II cells causes impaired embryonic development:

Our analysis using *Prkci* global knockout mouse model established the importance of PKCλ/ι signaling in mouse labyrinth development in early stages in placenta primordium. The global knockout embryos show lethality at around embryonic day 9.5. Our analysis with the *Prkci* conditional knockout model also revealed that the *Prkci fl/fl; Gcm1 cre* embryos dies in utero. We could not detect any live born pups having homozygous deletion of *Prkci* in the Gcm1 expressing cells of the placenta. We next wanted to understand at what stage of placental development are these embryos undergoing arrest in development. In order to understand the timing of in utero death, we carefully analyzed Gcm1 expression in the developing placenta.

Gcm1 expression has been detected as early as E7.5 where it is expressed in a few clusters of chorionic cells. After chorio-allantoic attachment takes place at E8.5, Gcm1 expression is expanded, separated by non-expressing cells and marks the points for invagination and initiation of labyrinth branching morphogenesis. Later on, Gcm1 is expressed all throughout the gestation days till E 17.5 (Basyuk et al., 1999a). Hence, we decided to analyze the embryos starting at day 7.5, 8.5 and 9.5, as we believe that PKCλ/ι signaling is most important at this initial stage of placental labyrinth morphogenesis based on our previous studies.

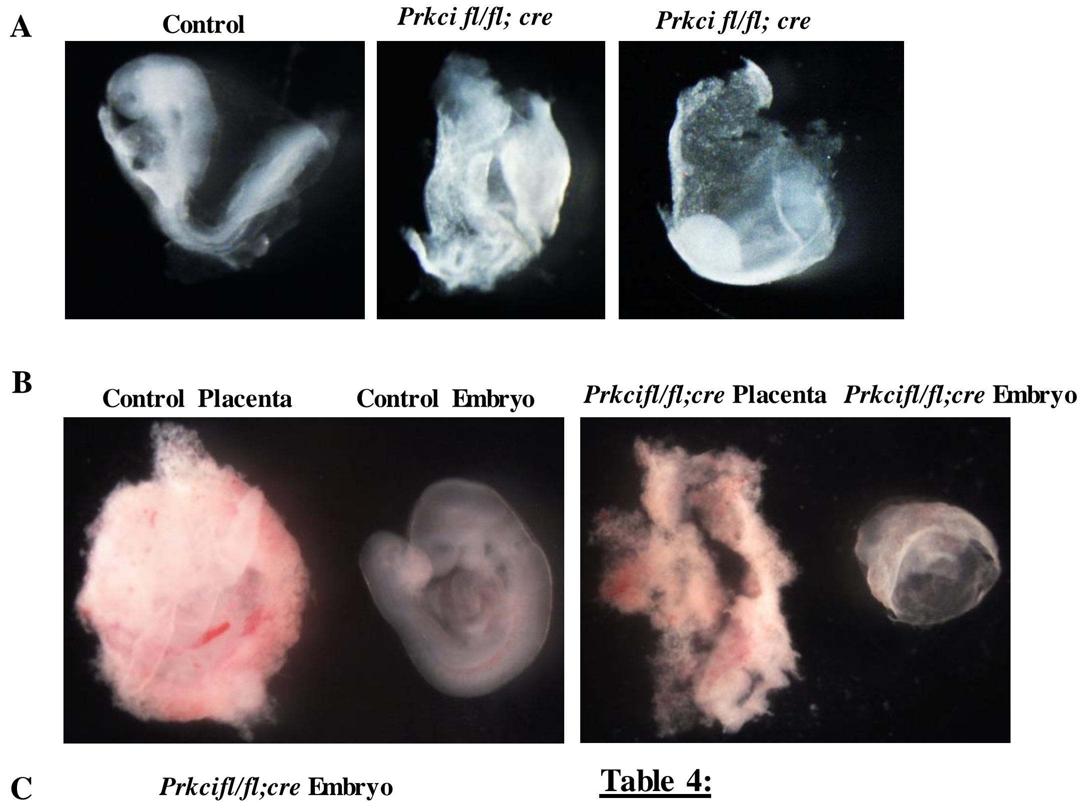
We set up matings of *Prkcifl/+; Gcm1 cre* (M) and *Prkcifl/fl* (F) and isolated embryos at E8.5 and E9.5. Our preliminary analysis at E8.5 so far reveals that deletion of *Prkci* in the *Gcm1* expressing cells at this early stage impairs embryonic development. This stage corresponds to Theiler stage 12 of development where the optic placode is visible in the embryos, which later indents and becomes optic pits. The rudimentary heart starts to develop at this stage. Foregut pocket appears, and the first appearance of 1-7 somite pairs. We could observe optic pit, the embryo hasn't

undergone turning event yet and neural tube closure is evident in some of the embryos (Figure 2A, left panel). In some of the embryos we observed, arrested neural tube closure, no visible optic pit, no visible somite formation, which indicated an impaired embryonic development (Figure 2A, middle and right panel).

We also analyzed embryos at E 9.5 of development using *Prkcifl/+; Gcm1 cre* (M) and *Prkcifl/fl* (F) for mating. At this stage, we observed that some of the *Prkcifl/fl; Gcm1 cre* embryos appeared to be arrested in development and resembled E 8.5 stage embryos (Figure 2B, right panel) with incomplete neural tube closure, impaired somite development, impaired turning of the embryo (Figure 2C). There was also no visible limb bud formation and rudimentary heart development. This very preliminary set of evidences shows that loss of PKCλ/ι signaling in the initiation phase of placenta primordia development results in impaired embryonic patterning. PKCλ/ι signaling in the labyrinth trophoblast is important for the proper development of the embryo proper. Loss of PKCλ/ι signaling in the Gcm1 expressing SynT-II cells results in impaired placental development (Figure 2B, right panel) which eventually affects embryonic patterning and development (Figure 2C).

FIGURES

Figure 2:



C ***Prkcifl/fl;cre* Embryo**

Table 4:

DOB of litters	Total No. of pups	Prkcifl/fl;Gcm1 cre	Other genotypes
10/23/19	7	0	7
11/14/19	7	0	7
12/06/19	3	0	3
1/21/20	4	0	4
12/06/19	3	0	3
12/30/19	2	0	2
1/8/20	5	0	5
1/31/20	3	0	3

Figure2: Loss of PKCλ/ι signaling in *Gcm1* cells of the labyrinth results in impaired embryonic and placental development: A- E8.5 control and *Prkci* KO embryos. B- E9.5 control and *Prkci* KO placenta and embryo morphologies show impaired placental and embryonic development. C- Mutant embryo morphology showing impaired development. Table1- Table showing number of post-natal pups analyzed across different litters indicating embryonic lethality of *Prkcifl/fl; Gcm1 cre* pups.

Chapter 4: Conclusion and future direction

In this study we have performed a comprehensive analysis to understand one of the most underrated but essential organ for wellbeing later in life- the placenta. Placenta has been referred to as the "the forgotten organ" since a lot of earlier studies involving mouse embryonic lethal mutants never analyzed the status of the placenta. A recent study, which conducted screening of 103 knockout mouse lines having embryonic lethality, revealed that 68% of those mutants have associated placental dysmorphologies (Perez-Garcia et al., 2018). The underlying signaling mechanisms in placental and embryonic development in humans, remains unexplored and with ethical restrictions binding use of human embryo and placental tissues makes research very difficult if not impossible.

However, in this study, we have shown the importance of a protein kinase C isoform, atypical PKCλ/ι signaling in maternal-fetal exchange surface development. PKCλ/ι was earlier known for its association with cell polarity complex, and overexpression in different cancer subtypes. In this study, our results show the importance of PKCλ/ι in the establishment of SynT, which form the maternal fetal exchange interface in mouse and humans. We have shown PKCλ/ι dependent evolutionary conserved signaling mechanism for the establishment of maternal-fetal exchange interface. Our analyses indicate that PKCλ/ι signaling is essential for maintaining the expression of PPARG/GATA2 signaling axis which in turn regulates GCM1/DLX3 mediated establishment of SynT in mouse and human. However, we need to understand how a protein kinase C isoform transcriptionally regulates GATA2/PPARG, which are both transcription factors. We believe that the answer lies within the kinase activity of PKCλ/ι.

We are currently trying to understand the essential regulators directly involved in PKCλ/ι signaling using a phospho-proteome analysis approach. We have earlier performed reversed phase protein array (RPPA) analysis in collaboration with the RPPA core in MD Anderson. However, the dataset

obtained is biased and only contains very few protein kinases and fails to provide an unbiased holistic picture of the signaling molecules regulated by PKCλ/ι. We are currently trying to understand the kinome profile of PKCλ/ι dependent molecular mechanisms with the help of Proteomics core in KUMC, which will reveal direct downstream targets of PKCλ/ι signaling and focus on the upstream regulators as well. This approach to understand the proteome profile of PKCλ/ι signaling will provide us the key targets of PKCλ/ι in mouse as well as in human trophoblast stem cells.

We have also found in our analyses using miscarriage placenta tissues, obtained after approval of patients and following IRB guidelines by the Institution that the expression of PKCλ/ι is significantly downregulated in some of these samples compared to age-matched control consented terminated placental samples. Our analyses also indicate that human trophoblast stem cells established from these miscarriage samples also has reduced PKCλ/ι expression compared to the cell lines established in the previous study (Okae et al., 2018) . About 15-20% of pregnancies result in miscarriage. Recurrent pregnancy loss (RPL) of unknown etiology affects about 1% of the women where they suffer from two or more consecutive miscarriages, however the underlying causes still remains unknown. Although our sample size and results show a very preliminary evidence of correlation between expression of PKCλ/ι and recurrent miscarriage, nevertheless, it will be interesting to further analyze more placenta samples obtained from RPL cases and analyze PKCλ/ι dependent molecular signaling mechanisms. Also identifying *PRKCI* gene mutations in these RPL samples can provide us important knowledge about the essentiality of PKCλ/ι signaling in pregnancy and whether it can be targeted as an early prognostic marker to detect possible RPL and hence counseling women with high probability of spontaneous pregnancy loss.

Our overall study reveals a small bit of the puzzle; most of which remains ‘a black box’. More research to understand the signaling pathways essential for placentation is important as proper development of the maternal-fetal interface dictates life-long health related outcomes. Recent research suggests that placenta and cardiovascular health of the baby are inter-dependent of each other. Improper placenta development during the gestation days can lead to cardiovascular diseases manifested later in adult life. Hence, research regarding the placenta should be permitted and researchers should have access to the placental tissue without unnecessary ethical restrictions that curbs the freedom of scientists to perform research in a collaborative and free world.

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