Atypical Protein Kinase C isoforms maintain human placental syncytiotrophoblast polarity

by

Khushali Patel

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences - Obstetrics and Gynecology
University of Alberta

Abstract

The human placental surface is lined by a giant multinucleate epithelial cell – the syncytiotrophoblast (ST). The ST acts a maternal-fetal interface and aids in the exchange of gas, nutrient exchange, and acts as a transient endocrine organ. This ST layer is formed and maintained by the underlying mononuclear proliferative villous cytotrophoblasts (vCTs). The ST is a highly polarized epithelium with dense microvilli emerging from its apical surface bathed in the maternal blood in the intervillous space.

Microvilli (MV) are a hallmark of established apical-basal polarity in epithelial cells. These MV are membrane protrusions supported by an F-actin core. MV structure is dynamically maintained by constant actin treadmilling and cytoskeletal linker proteins belonging to the Ezrin/Radixin/Moesin (ERM) family aid in anchoring the F-actin core to the cell membrane. Previous electron micrographs reveal abundant expression of ezrin at the ST apical membrane. When phosphorylated, ezrin transforms from a dormant configuration to active configuration where it's C-terminal end binds to the F-actin core and the N-terminus binds to the cell membrane.

Polarity is the asymmetric distribution of biomolecules in a cell. Recent studies have revealed that mRNA transcribing for various transporters, vesicles and hormones are specifically expressed at the ST apical surface. Studies have also revealed that extracellular vesicles containing active biomolecules such as DNA, RNA and protein are actively released from the ST microvillar region. Thus, the ST and its microvilli seem to play a critical role in the normal functioning of the human placenta. However, active maintenance of polarity is critical for normal cellular homeostasis. Yet to date, no studies have investigated the maintenance of polarity in this highly polarized epithelium.

Atypical Protein Kinase C (aPKC) isoforms are a part of the Par complex- an evolutionarily conserved regulator of apical-basal polarity. It is known that aPKC along with the scaffolding

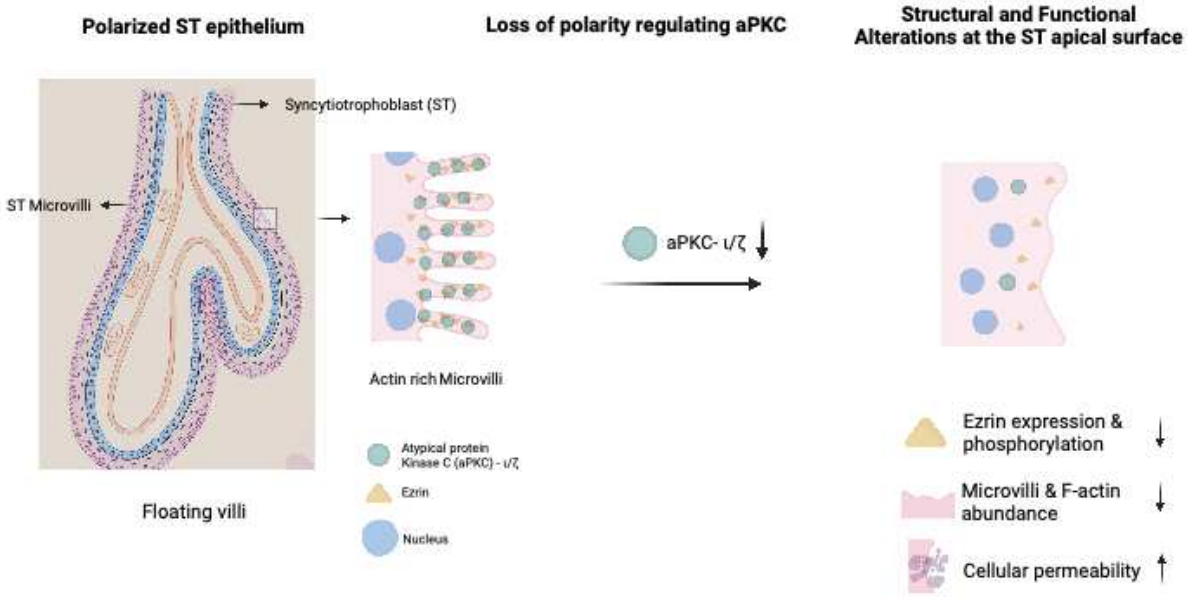
proteins Par-3 and Par-6 localize apically and determine the apical domain in epithelial cells. In humans, two full length isoforms – aPKCι and aPKCζ are expressed along with a constitutively active brain-specific N-terminal truncated aPKCζ isoform: PKMζ. APKCι/ζ isoforms have been shown to maintain polarity in various human epithelial cells. In the mice intestinal microvilli, aPKCι has been shown to regulate ezrin phosphorylation. While aPKCλ/ι knockout mice are known to be embryonically lethal at E9.5, aPKCζ knockout mice have no embryonic phenotype. However, later it was revealed that aPKCζ can partially compensate for the aPKCλ/ι knockout phenotype. Recent studies reveal the expression of aPKCι in human trophoblasts, and the critical role it plays in regulating trophoblast stem cell (TSC) to ST differentiation. However, there is a lack of literature that takes the compensatory nature of aPKCs into consideration and investigates the expression of multiple aPKC isoforms in the human trophoblasts.

Data presented in this thesis reveals the expression of a novel aPKC isoform – aPKCζ III along with the full length aPKCι and aPKCζ isoforms in the human trophoblasts. APKCζ III is a N-terminal truncated isoform derived from an alternate promoter region of the *PRKCZ* gene. However, not much is known about the expression and function of this novel aPKC isoform in the human placenta.

The data presented in this thesis also identify the apical localization of the aPKC isoforms along with ezrin and that aPKC kinase activity is partially responsible for ezrin phosphorylation at the ST apical surface. Inhibition of total aPKC kinase activity leads to a significant region-specific loss of ST apical F-actin and microvilli. Additionally, the loss of either one of the aPKC isoforms is enough to cause structural and functional alterations in the ST.

In conclusion, the results presented in this thesis reveal a critical yet functionally redundant role of aPKC isoforms in maintaining ST apical polarity.

Graphical representation of the abstract



Created with BioRender.com

Preface

This thesis is an original work by Khushali Patel. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Human Research Ethics Board (Molecular regulation of early human placental development Pro00089293; Placental and vascular mechanisms of preeclampsia and intrauterine growth restriction; Pro00088052). Human placental samples were collected by methods approved by the University of Alberta human research ethics board. First trimester placental tissue was obtained from elective pregnancy terminations following informed consent from the patients. Term placental tissue was collected from uncomplicated pregnancies after cesarean delivery without labor. Whole mouse brain (3-month old C57BL/6J) collection was approved by the University of Alberta Research Ethics Office in accordance with the Canadian Council for Animal Care Policies.

Chapter 3 of this thesis has been published as: Sumaiyah Shaha, Khushali Patel, Saba Saadat, Sareh Panahi, Monique M. de Almeida, Anastassia Voronova, Meghan Riddell, Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c,Placenta, Volume 119, 2022, Pages 39-43, ISSN 0143-4004, https://doi.org/10.1016/j.placenta.2022.01.015.

I was responsible for performing siRNA knockdown experiments and all western blot analyses. S.Panahi and S.Shaha performed trophoblast isolation and trophoblast culture. S.Shaha performed the RT-PCR. A.Voronova and M.M. de Almeida provided the mouse brain and contributed feedback on the manuscript. M. Riddell conceptualized the project,

conducted the immunofluorescent staining, and wrote the manuscript. S.Shaha and I contributed with data collection, analysis, and manuscript edits.

Chapter 4 of this thesis has been published as part of pre-print : K.Patel*, J.Nguyen*, S.Shaha, A. Zubkowski, M. Riddell; “Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal-fetal interface”, *Biorxiv 2022.* doi: https://doi.org/10.1101/2022.06.30.498172

I was responsible for the collection of placental tissue, placental explant culture experiments including inhibitor treatment and optimizing siRNA knockdown treatment and conducting immunofluorescence experiments, and ELISA assays. I also performed electron microscopy sample processing and imaging. I was responsible for data collection, analysis, and contributed to manuscript composition and editing. J. Nguyen conceptualized, optimized, designed, and performed the dextran uptake assays. She also performed explant tissue culture treatments. She also contributed to the manuscript composition and editing and made the figures. S. Shaha performed trophoblast isolations and trophoblast culture. A. Zubkowski assisted in the collection of preliminary data and performed immunofluorescent staining and western blotting analyses. M. Riddell conceptualized the study, carried out explant cultures, immunofluorescent staining, and dextran uptake assays. She also composed the manuscript and supervised the project.

The literature review in Chapter 1, western blot data collection and analysis in Chapter 3, data collection and analysis in Chapter 4 as well as concluding analysis and discussion presented in Chapter 5 are my original work.

Dedication

The work represented in this thesis is dedicated to my Mom, Khyati, Dhruv, Maa-Paa and my grandparents.

Acknowledgements

I would like to take this opportunity to extend my gratitude to all the people who played a significant role in helping me achieve this milestone.

Firstly, I would like to thank my mentor Dr. Meghan Riddell for the example she has set for all the Riddell lab students. Meghan has been my role model since the day I first met her for my MatCH program rotation. Her dedication towards her work has always inspired me and I hope a part of it reflects in this thesis. Without her tireless support, patience, and guidance, I wouldn't have reached here. She has always inspired me not just academically, but also in the way she balances her personal and professional life. Her ability to think critically and her 'out-of-the-box' questions is something I truly want to imbibe and reflect in my academic career. She has constantly pushed me towards growth and motivated me through the most difficult time of my Masters journey. Thank you for inspiring me and believing in me Meghan. I will always carry that spark for science and research that you have instilled in me. I truly admire your enthusiasm and passion for science.

Secondly, I would like to express my gratitude towards my supervisory committee members – Dr. Davidge and Dr. Hughes, whose positive criticism, mentoring and support has been a critical part in this journey. I would also like to thank my program coordinator, Dr. Hemmings for her support and guidance. I would also like to extend my gratitude to Dr.Morrish for being on my examining committee.

I would like to thank my very kind and talented lab colleagues – Sumaiyah, Bethan, Jasmine and all the members from Riddell lab, Hemmings lab, Davidge lab, Bourque lab and Olson lab whose friendship, support and feedback has been an important part of my journey. The impromptu coffee runs, Christmas egg-nogg lattes, lab lunches have made this monotonous science life a little more fun and energetic.

I would especially like to thank our research nurse Maya Henriquez and Donna Dawson for their amazing ability to multitask and coordinate the collection of placentas. I would also like to thank Dr. Kacie Norton and Arlene Oatway of the Biological Sciences Imaging Facility, University of Alberta, for their expert technical advice on electron microscopy.

I am thankful to the following funding agencies as without their support, the work presented in this thesis wouldn't have been possible: the maternal and child health program (MatCH), Canadian Institutes for Health Research (CIHR), Women and Children's Health Research Institute (WCHRI), Alberta Women's Health Foundation (AWHF) and Stollery Children's Hospital Foundation (SCHF). I would like to express my gratitude to the Faculty of Medicine and Dentistry and University of Alberta for providing me an incredible platform to pursue my dreams.

I would like to take a moment and appreciate the constant support, love, and patience that Dhruv has shown towards me. Thank you, Dhruv, for pushing me the most and not letting me give up when life threw lemons at me. You have been my biggest fan and my worst critic. I hope now you can explain to people what I did my Masters in and you read and understand at least a page of this thesis. Also thank you Mom and Maa-Paa for being my biggest cheerleaders.

Lastly, I would like to thank my family and friends for always supporting and believing in me.

Table of Contents

Abstract ..... *ii*

CHAPTER 1 – GENERAL INTRODUCTION **1**

1.1 Human Placental Structure and Cell Types 1
1.2 Placental Villi and Villous Branching 2
1.3 Trophoblasts 5
1.3.1 Villous Cytotrophoblasts (vCTs) 5
1.3.2 Differentiation of vCTs into ST 6
1.3.3 Syncytiotrophoblast (ST) 7
1.3.3.1 ST structure and its microvilli 7
1.3.3.2 ST functions 9
1.4 Polarity 13
1.4.1 The Par Complex 13
1.4.2. The Crumbs Complex 16
1.4.3. The Scrib Complex 16
1.4.4 Polarity complex expression and function in the human placenta 18
1.5 Rationale 20
Thesis Objectives and Hypothesis 21
References 23

CHAPTER 2 – MATERIALS AND METHODS **31**

2.1 Tissue collection 31
2.2 Trophoblast isolation and culture 31
2.3 Floating placental explant culture and treatments 32
2.3.1 APKC inhibitor treatment 32
2.3.2 PRKCI and PRKCZ targeted siRNA knockdown treatment 33
2.4 Dextran Uptake Assay 34
2.5 Immunohistochemistry and image analysis 34
2.5.1 Antigen Retrieval 35
2.5.2 Immunofluorescence 35
2.5.3 Image capture 36
2.5.4 Image quantitation 37
2.6 Electron microscopy 37
2.6.1 Scanning electron microscopy (SEM) 37
2.6.2 Transmission electron microscopy (TEM) 38
2.7 Western Blotting 38
2.8 RNA-isolation and RT-PCR 40
2.9 ELISA assays 40
2.10 Statistical Analysis 41

CHAPTER 3 – HUMAN PLACENTA AND TROPHOBLASTS SIMULTANEOUSLY EXPRESS THREE ISOFORMS OF ATYPICAL PROTEIN KINASE-C **42**

3.1 INTRODUCTION **42**

3.2 Objective 43
3.3 Results 44
3.4 Discussion 53
3.5 References 57

CHAPTER 4 – APKC ISOFORMS MAINTAIN ST APICAL SURFACE 59

4.1 Introduction 59
4.2 Hypothesis 61
4.3 Results 61
4.4 Discussion 82
4.5 References 88

CHAPTER 5 – DISCUSSION 91

5.1 Summary of the key findings 91
5.2 Overall significance 93
5.4 Future Directions 96
5.4.1 APKC-Actin Pathway 96
5.4.2 Is polarity loss pathologically relevant? 97
5.5 References 99
Bibliography: 102
Appendix 1 : Copyright license for Fig 1.2 117
Appendix 2 : Copyright license for Fig 1.3 118

List of Tables

TABLE 2.1 - TARGET SEQUENCE FOR SIRNA TARGETING PRKCI AND PRKCZ GENE 34
TABLE 2.2 - LIST OF PRIMARY ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY (IHC) 36
TABLE 2.3 - LIST OF PRIMARY ANTIBODIES USED FOR WESTERN BLOTTING 39
TABLE 2.4 - PRIMER SEQUENCES FOR PRKCZ ISOFORMS 40

List of Figures

Graphical representation of the abstract iv
Figure 1.1: Human Placental Structure 3
Figure 1.2: Human Placental Villi Branching 5
Figure 1.3: Transmission electron microscopy of human ST apical surface 12
Figure 1.4: Schematic of aPKC isoforms expressed in humans 15
Figure 1.5: Schematic of apical-basal determining polarity complexes expressed in mammalian epithelial cells 18
Figure 3.1: Human placental trophoblasts in early and late gestation express aPKC-ι isoform. 45
Figure 3.2: Human placental trophoblasts in early and late gestation express aPKC-ζ isoform. 46
Figure 3.3: Western blotting of first and third trimester placenta and isolated primary cultured CT and ST show a 55kDa and 75kDa band with anti-aPKC-ζ targeting antibodies. 48
Figure 3.4: siRNA KD validation of anti-aPKC-ζ antibody. 49
Figure 3.5: PRKCZ isoform specific primers amplify isoform 1 mRNA in villous trophoblasts and whole placental lysate. 51
Figure 3.6: PRKCZ isoform specific primers amplify isoform 2 mRNA only in the human brain. 52
Figure 3.7: PRKCZ isoform specific primers amplify isoform 3 mRNA in villous trophoblasts and whole placental lysates. 53
Figure 3.8: Schematic representation of aPKC isoforms in humans 55
Figure 4.1: aPKC-ι colocalizes strongly with ezrin at the ST apical surface in 9-12 weeks and 37-40 weeks ST. 63
Figure 4.2: aPKC-ζ strongly colocalizes with ezrin at the ST apical surface in 9-12 weeks and 37-40 weeks ST. 64
Figure 4.3: aPKC activity regulates ST apical ezrin activation and abundance in 9-12 weeks placental explants. 66
Figure 4.4: aPKC activity regulates ST apical ezrin activation and abundance in 37-40 weeks placental explants. 67
Figure 4.5: aPKC inhibitor treatment alters apical F-actin abundance in both first trimester (9-12 week) and term (37-40 weeks) placental explants. 68
Figure 4.6: PRKCI targeting siRNA knockdown efficiency in first trimester explants (9-12 weeks) 71
Figure 4.7: PRKCZ targeting siRNA knockdown efficiency in first trimester explants (9-12 weeks) 72
Figure 4.8: PRKCI+PRKCZ targeting siRNA knockdown efficiency in first trimester explants (9-12 weeks). 73
Figure 4.9: siRNA knockdown targeting either PRKCI or PRKCZ isoforms alter apical F-actin abundance in 9-12 week placental explants. 74
Figure 4.10: Second set of siRNA targeting either PRKCI or PRKCZ isoforms decreases apical F-actin abundance in 9-12 week placental explants. 75
Figure 4.11: SEM images of 9-12 week placental explants treated with aPKC inhibitor reveal decreased apical microvilli. 77
Figure 4.12: aPKC inhibition leads to simplification of ST apical membrane, decreased cytoplasmic density 78
Figure 4.13: aPKC inhibition permeabilizes ST to 10000 MW neutral compound. 79
Figure 4.14: siRNA knockdown targeting either PRKCI or PRKCZ isoforms permeabilizes the ST to 10000 MW neutral compound. 80
Figure 4.15: Extracellular release of human chorionic gonadotropin b sub-unit increases after 6 hours of aPKC inhibitor treatment. 82

List of Abbreviations

ABBREVIATION	FULLFORM
ABC	ATP-binding cassette
Ang-2	Angiopoietin 2
ANOVA	Analysis of variance
aPKC	Atypical Protein Kinase C
BCA	Bicinchoninic acid
BM	Basal membrane
CO_2	Carbon dioxide
CRB complex	Crumbs complex
CT/ sCTB	Cytotrophoblasts
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ERM	Ezrin/Radixin/Moesin
EVTs	Extravillous trophoblasts
F-actin	Filamentous actin
FLRG	Follistatin related gene
GSDMD	Gasdermin D
hCG	Human chorionic gonadotropin
HMDS	Hexamethyldisilazane
hPL	Human placental lactogen
INHA	Inhibin A
kDa	Kilo-Dalton (unit of molecular weight)
LatA	Latrunculin A
MLKL	Mixed lineage kinase domain-like protein
mRNA	Messenger ribonucleic acid
MV	Microvilli
MVM	Microvillar membrane
O_2	Oxygen
Par 3	Partition defective gene 3
Par 6	Partition defective gene 6
PBS	Phosphate buffered saline
PCNA	Proliferation cell nuclear antigen
pCT	Progenitor cytotrophoblast
PE	Preeclampsia
PGH	Placental growth hormone
PIGF	Placental growth factor

RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Sdt	Stardust
SEM	Scanning electron microscope
siRNA	Small interfering Ribonucleic acid
SLC	Solute carrier
ST/SYN	Syncytiotrophoblast
STBEV	Syncytiotrophoblast derived extracellular vesicles
TE	Trophectoderm
TEM	Transmission electron microscope
TSC	Trophoblast stem cells
vCT	Villous cytotrophoblast
WGA	Wheat germ agglutinin

Chapter 1 – General Introduction

A series of cleavage divisions of the zygote initiates mammalian development. Subsequently at the blastocyst stage of development, cells progressively differentiate from each other to establish embryonic and extra-embryonic tissues, which is essential for implantation in the uterus and further embryonic development(1). Three lineages have to become established by the time of embryo implantation: the trophectoderm (TE), which will generate the placenta, the epiblast, which will form the embryo proper, and the hypoblast, which will form the yolk sac (1). The placenta is a fetally derived transient organ that serves as the exchange interface between the mother and the fetus facilitating the transfer of oxygen, nutrients and waste thereby controlling fetal growth. It acts as an endocrine organ that secretes pregnancy sustaining hormones as well as an immunologic barrier that protects the fetus from the maternal immune response. Thus, making the placenta one of the first crucial organs to develop in order to sustain a healthy pregnancy (2).

1.1 Human Placental Structure and Cell Types

The human placenta is a discoidal shaped organ that invades the maternal uterine tissue. It is arranged in a highly branched villous tree-like structure where progressively smaller villi are emanating from larger villi. These villous trees are bathed in the maternal blood of the intervillous space, and the placenta is anchored to the maternal uterine decidual tissue through the anchoring villi (See Fig. 1.1). Placental villous tree development is necessary for continued fetal growth and well-being (3). There are three major cell types in the placenta: epithelial cells, vascular cells, and stromal cells. The placental epithelial cells - the trophoblasts; are the first committed cell lineage in an embryo derived from TE and the only placental cells in direct contact with maternal blood

and tissue. Trophoblasts are derived from trophoblast stem cells (TSC); bipotential cells that give rise to the progenitor cytotrophoblast cell (pCT) populations which can differentiate into two trophoblast lineages: the villous lineage and the extravillous lineage (4). The villous lineage comprises the villous cytotrophoblasts (vCT) that fuse together and differentiate to form the syncytiotrophoblast (ST), lining the placental surface (5). The ST is in direct contact with the maternal circulation and is responsible for most of the nutrient transport across the placenta for maintaining a healthy pregnancy (6). The extravillous lineage comprises the extravillous trophoblasts (EVTs) which invade the maternal uterine decidual tissue and aid in the maternal spiral artery remodeling (2).

Blood vessels are a key component of placental villi. Vascular cells of the placenta include endothelial cells and pericytes. The core of the placental villi is comprised of stromal cells, mainly fibroblasts and macrophages (known as Hofbauer cells in the placenta). Stromal cells are also crucial for the process of angiogenesis, production of growth factors as well as immune response (2).

1.2 Placental Villi and Villous Branching

Villous development initiates 12-18 days post conception. Based on the villous structure of the placenta, villi branch out extensively and form smaller floating villi that fills up the intervillous space further increasing the surface area for exchange. When the extraembryonic mesoderm and blood vessels originating from the allantois (progenitor tissue of the umbilical cord) infiltrate the primary villi cores proximally, it forms the chorionic villi. This villous core consists of stroma including immune cells and fibroblasts as well as blood vessels and is lined by a trophoblast

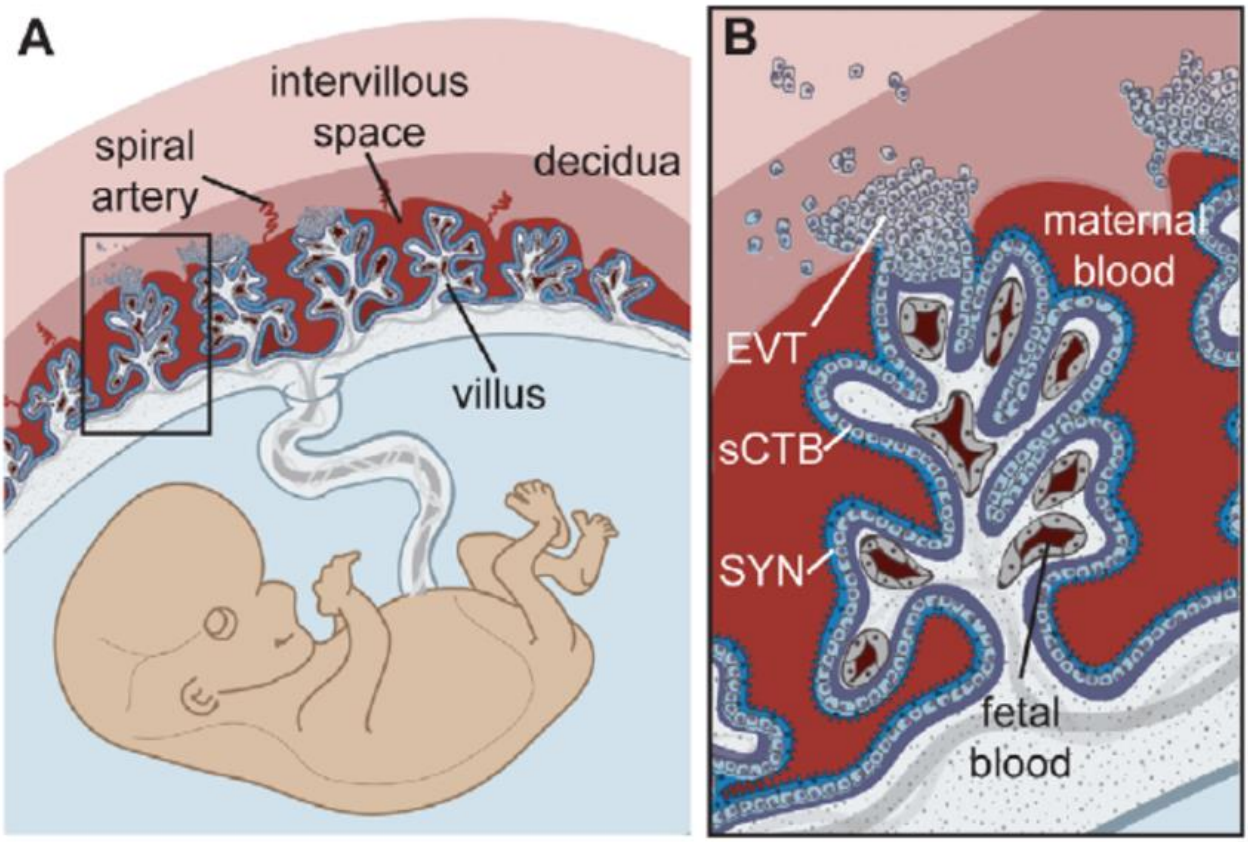


Figure 1.1: Human Placental Structure

(A) General schematic of the human placenta (B) inset image depicting the placental villus invading maternal uterine decidual tissue with different trophoblast cell types. SYN – syncytiotrophoblast, sCTB – cytotrophoblasts, EVT – extravillous trophoblasts. Adapted from Zeldovich et.al (2011). Invasive Extravillous Trophoblasts Restrict Intracellular Growth and Spread of Listeria monocytogenes. PLoS pathogens. © 2011 Zeldovich et. al

bilayer containing an inner vCT layer and an outer ST layer separated from the underlying stroma via a basement membrane. It is assumed that in the villous tree hierarchy, the most proximal parts positioned near the chorionic plate are the oldest ones, whereas the most peripheral branches are the recently developed ones (7).

First trimester placental villi can be categorized into primary villi consisting of only vCTs and the ST, secondary villi consisting of trophoblasts as well as extraembryonic mesenchyme invading the primary villus core, and tertiary villi which also contain capillaries (8). The first generation of these tertiary villi is known as the mesenchymal villi, and these lay the structural foundation for the morphological prerequisites for an effective maternal-fetal exchange surface (See Fig. 1.2). By the end of first trimester, the placenta expands through the formation of villous sprouts on the existing villi, thus there is an abundant pool of mesenchymal villi that can further form specialized villous types throughout the rest of the gestation. Tertiary villi undergo further differentiation processes to form different types of villi varying in structure and function throughout the second and third trimesters (2).

Several subtypes exist only beyond the first trimester. For example, as seen in Fig 1.2, stem villi are one of the largest villi (100-3000 μm in diameter) of the tertiary villous subtypes. They are thought to aid in mechanical stabilization and placental blood flow due to the unique presence of myofibroblastic sheath (2,9). Additionally, intermediate villi are large bulbous (100-500 μm in diameter) villi which are further categorized into peripheral, bulbous immature intermediate villi and elongated, slender mature intermediate villi which further produce the terminal villi (2). Terminal villi are grape-like structures characterized by a high degree of capillarization and are linked to stem villi by intermediate structures (Fig 1.2). In the terminal villi, the fetally derived capillary vessels are separated from the ST by a thin basement membrane with minimal maternal-fetal diffusion distance, which enhances the diffusive exchange at the terminal villi. Due to their structure terminal villi are presumed to be the primary site of electrolyte transfer, as well as O_2, CO_2 and nutrients between the mother and fetus (2,10,11). In term placenta, terminal villi decrease in size with less stroma and a discontinuous vCT layer (2).

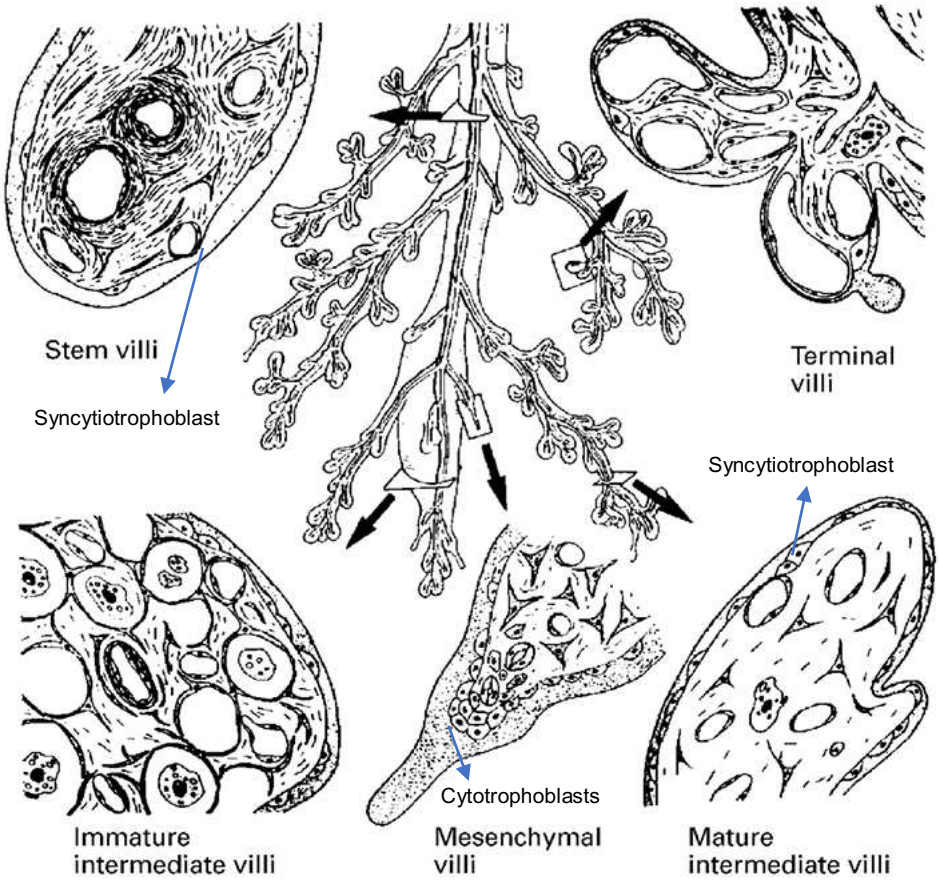


Figure 1.2: Human Placental Villi Branching

General schematic of the human villus branching depicting the Stem villi, Intermediate villi, Mesenchymal villi and Terminal villi lined by syncytiotrophoblast, cytotrophoblasts as well as blood vessels and capillaries. Adapted from Haines & Taylor obstetrical and gynaecological pathology. Permission obtained from Rightslink. Certificate attached in Appendix 1.

1.3 Trophoblasts

1.3.1 Villous Cytotrophoblasts (vCTs)

As mentioned above, the cytotrophoblasts or CTs are proliferative mononuclear cells that are derived from the TSCs. The more primitive CT progenitors are thought to reside in the anchoring

villi while the vCTs reside under the ST basal membrane (2). The proliferative CTs differentiate into vCTs that reside on top of the basement membrane that separates the underlying stroma from the trophoblastic epithelial cell layer. Transmission electron microscopy images reveal that ultrastructurally, vCTs contain fewer mitochondria and rough endoplasmic reticulum, however abundant polyribosomes (7). With increasing gestational age, these cuboidal vCTs become less dense and thin (2,12,13). VCTs have also been shown to express E-cadherin, an adherens junction protein that helps the mononuclear vCTs to form a tight connection with surrounding vCTs. E-cadherin expression that decreases as the vCTs fuse together to form the form the overlying layer of ST, hence strong junctional expression of E-cadherin is often used as a marker of vCTs (14–17).

1.3.2 Differentiation of vCTs into ST

The *in vitro* stimulation of trophoblast differentiation from primary human vCTs to a ST phenotype is revealed to be a highly complex and orchestrated process. It involves morphological changes that promote the villous CTs to proliferate and then exit the cell cycle followed by fusion and form the syncytium which requires the fusion of CT and ST membranes usually mediated by cellular fusogens such as syncytins (10,18–20). Additionally, cytoskeletal changes also occur to form extensive microvilli on the ST apical surface (21). This is coordinated with simultaneous molecular level changes that promote biochemical differentiation during which the cellular organelles of the CT are spatially reoriented in the ST in addition to the repression of CT genes involved in proliferation (10). Concomitantly, the newly fused ST begins to express genes involved in hormone synthesis and its secretion, substrate transport, metabolism, and other functions of terminally differentiated ST (15). Various studies have shown that the two phases of differentiation

are coupled and that morphological differentiation is necessary before the cells can undergo biochemical differentiation (19).

Functionally, in addition to forming and maintaining the ST layer, cytotrophoblasts have also been shown to secrete angiogenic factors such as Angiopoietin 2 (Ang-2), Angiogenin, Placental growth factor (PlGF) and several others that are critical for placental vascular development (22).

1.3.3 Syncytiotrophoblast (ST)

As mentioned above, ST formation and maintenance is attained by the differentiation and fusion of the underlying proliferative mononuclear CTs. Various studies have investigated whether cellular replication of the ST occurs, however despite there being abundant intracellular organelles present in the ST, there has been no immunohistochemical evidence of proliferation markers such as Ki67 and proliferation cell nuclear antigen (PCNA) (23). Thus, ST must be regularly replenished by controlled fusion and differentiation of underlying CTs throughout the pregnancy due to its highly regulated turnover (24).

1.3.3.1 ST structure and its microvilli

Uniquely, the ST is a single giant multinucleate cell that has an estimated surface area of $12m^2$ by term (8). The ST has a highly defined convoluted apical surface with microvilli bathed in the maternal blood in the intervillous space. Microvilli are membrane enclosed protrusions emerging from the apical surface which are supported by an underlying filamentous actin (F-actin) core. It is a unique human epithelium for its lacking lateral membranes (2,8).Critically, as mentioned

previously, the ST in terminal villi is separated from the fetal capillaries by a very thin basement membrane thus enhancing the maternal-fetal exchange at the ST surface. Importantly, the extensive and continuous ST layer has region specific structure and function revealed by the asymmetric distribution of biomolecules in transmission electron microscopy (TEM) images (2). Based on these TEM images, Dempsey and others subdivided the syncytiotrophoblast into three zones: the absorptive, secretory, and basal zone. The outer absorptive zone designated as the apical surface is rich in cytoskeleton as well as organelles required for vesicular uptake such as small and large coated vesicles. The middle secretory zone is rich in rough endoplasmic reticulum that helps in secretion of hormones. This middle zone is also shown to contain most of the organelles and the thickness and structural variability in this middle zone is associated with regional specialization. The basal zone has fewer organelles similar to those found in the vCTs (25). The asymmetric reorganization of biomolecules appears to aid in the proper functioning of the ST. TEM images show that the regions of the ST containing abundant rough endoplasmic reticulum and mitochondria are heavily involved in hormone synthesis and energy metabolism (2). While other areas abundant in smooth endoplasmic reticulum have been shown to be involved in lipid synthesis (2). This structural asymmetry of biomolecules and/or organelles is analogous to the functional specialization and compartmentalization thus leading to regionalization in the ST cell despite the lack of intracellular borders.

The presence of highly differentiated ST surface with its dense microvilli amplifies the apical surface area of the ST and is assumed to help with nutrient absorption. Apical microvilli also highlight that the ST is a highly polarized cell (Fig 1.3). In the intestinal brush border, microvilli have been associated with increase in the surface area of the intestinal plasma membrane for nutrient absorption but also to shed protein enriched vesicles in the gastrointestinal tract (26,27). Thus, ST microvilli not only seem to increase the surface area for enhanced transport mechanism

but may also be essential sites for the secretion of biomolecules critical for a healthy dialogue between the mother and the fetus.

It has been shown that the lipid composition of the microvillar membrane is involved in signal transduction as well as receptor mediated endocytosis (28). Importantly, it has also been shown that the microvillar membrane proteome physiologically adapts to cellular needs, making this site one of the most dynamic cellular structures (29). The underlying filamentous actin (F-actin) core is constantly treadmilling and a critical feature of microvilli is the lateral attachment of the F-actin core to the plasma membrane. This attachment has to sustain tension across the epithelium to maintain the microvillar integrity. To sustain this, activation of a cross linking protein of the Ezrin-Radixin-Moesin (ERM) family is necessary. These proteins serve to link the microvillar F-actin to the membrane (30). The ST has a densely branched microvilliated surface. Overall, this suggests that active maintenance of the ST microvilli is crucial for ST functional homeostasis. However, even though the ST is known to play a crucial role in transport and nutrient uptake, to our knowledge there is a paucity of data that studies the active maintenance of this highly polarized microvillar surface of the ST.

1.3.3.2 ST functions

ST is responsible for the synthesis and secretion of multiple hormones, such as human chorionic gonadotropin (hCG), human placental lactogen (hPL) placental growth hormone (PGH). The ST also produces a large number of growth factors and cell signaling molecules such as inhibin A (INHA), follistatin related gene (FLRG) and several others. (31–33). These hormones and signaling molecules are critical for the establishment of pregnancy as well as modulating the

maternal physiology in order to sustain the pregnancy (33). In addition, ST releases many types of extracellular vesicles (STBEV) including microvesicles and exosomes into the maternal circulation (34). These STBEVs contain various biologically active molecules such as RNA, proteins and lipids that interact with the maternal endothelial cells or leukocytes to mediate maternal-fetal communication as well as maternal immune response during pregnancy (34). Critically, the release of these extracellular vesicles is postulated to occur within the microvilli rich ST apical surface based on TEM observations (35,36). Thus, ST apical surface and its microvilli seem to play a critical role in the secretion of hormones and other signaling biomolecules.

Fetal development is entirely dependent on the maternal supply of nutrients (37). Transplacental transport involves varying mechanisms such as passive diffusion, pinocytosis, and facilitated and active transport via molecular transporter proteins which also support the barrier function of the placenta. The placenta is equipped with several membrane transporters that facilitate nutrient transport. Transporters belonging to the ATP-binding cassette (ABC) family mediate the distribution of various endogenous substrates, including steroid hormones, lipids, cholesterol, and factors involved in inflammatory and immunological responses (38). Various transporters of the solute carrier (SLC) transport family are also functionally expressed in the trophoblast cells, mediating the transport of amino acids, glucose, monoamines, organic cations and anions, and a wide range of drugs and toxins (39–42). Recent studies also show that placental transporters may regulate intracellular signaling as well as cell differentiation (38,43). Karahoda *et. al* recently performed an mRNA expression study to determine the expression and functional activity of various membrane transporters at varying trophoblastic differentiation state using term isolated trophoblasts. They reveal enhanced protein expression of membrane transporters involved in transplacental exchange of essential nutrients like cholesterol (ABCA1, ABCG1), iron (TfR1) and leucine (SLC3A2, SLC7A5) in syncytialized cells (44). Thus, the ST not only functions as an

endocrine organ, but also as a transport epithelium. Recently, the process of trophoblast syncytialization was associated with the enrichment of genes involved in amino acid transport (33). It was also shown that the process of CT differentiation into the ST primarily enhances key transporters for cationic and large neutral amino acids (SLC7A1, SLC7A6, and SLC7A9), while the expression of anionic and neutral amino acid transporters is not significantly affected (44). Thus, abnormal trophoblast differentiation could lead to nutrient transfer imbalance. Importantly, most of these transporters are found on the ST apical surface abundant in microvilli (45). Additionally, this polarized localization of the transporters helps in regulating the directionality of varying biomolecular flux across the placenta from both maternal and fetal side (45).

The ST also acts as a physical and immunologic barrier that separates the maternal and fetal circulation, thus protecting the fetus from infection as well as from rejection by the maternal immune system. Recent studies suggest that the placenta has an innate defense mechanism to protect against viral and protozoan invasion. These studies look at the vertical transmission of *L.monocytogenes, T.gondii, H.simplex* using human placental explants and they commonly reveal that while the ST prevented the invasion of these pathogens, mononucleur E-cadherin expressing cells were strongly infected by these pathogens (46–48). Robbins *et. al* show that E-cadherin acts as a portal for *L.monocytogenes* invasion in mononuclear trophoblasts (47,49). Typically, pathogens exploit receptors expressed at the intercellular junctions to breach the epithelial barriers (50,51). Thus, the unique lack of lateral domains in the syncytiotrophoblast as well as the scarcity of junctional proteins on its apical surface bathed in maternal blood could be one of the possible mechanisms by which it resists microbial invasion (50). However, syncytial resistance to microbial invasion via junctional protein independent mechanisms have also been shown suggesting that additional mechanisms may contribute to syncytial defenses (47,48). Additionally,

Zeldovich *et al* showed that actin depolymerization in primary human placental explant cultures led to increased *L.monocytogenes* invasion, suggesting that syncytial actin cytoskeleton may form a biophysical barrier against pathogen invasion (52). Thus, the unique structure of the laterally vast ST with its strong apical accumulation of the actin cytoskeleton might contribute towards its ability to act as an immunologic barrier.

Thus, the location, structure and functions of the ST makes it one of the most crucial components of the human placenta.

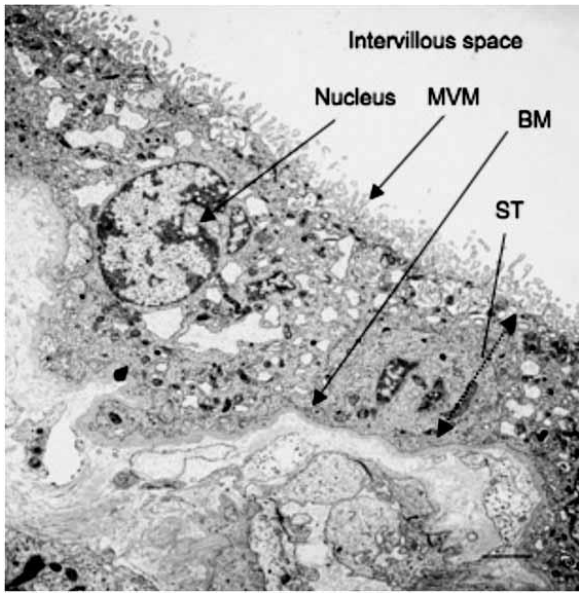


Figure 1.3: Transmission electron microscopy of human ST apical surface

TEM image revealing human ST surface with microvilli on its apical surface. ST – syncytiotrophoblast, MVM – microvillar membrane, BM – basal membrane. Modified from Jansson et. al (2009). The Role of Trophoblast Nutrient and Ion Transporters in the Development of Pregnancy Complications and Adult Disease. Current vascular pharmacology. Permission obtained from Rightslink. Certificate attached in Appendix 2.

1.4 Polarity

Cell polarity is defined as the spatial asymmetric distribution of cellular components including organelles and proteins within a cell. Epithelial cells are typically polarized to form distinct apical and basolateral domains. As introduced above, the ST uniquely lacks lateral membranes. Hence, it instead has an apical and basal surface.

Critically, as previously mentioned in 1.2.2, the process of fusion and differentiation of CTs into ST requires organelle and cytoskeletal reorganization allowing for the transition from an unpolarized CT progenitor to a highly polarized ST. Apical basal polarity is established and actively maintained via antagonism between apical and basal domain regulating polarity modules such as the Par complex, Crumbs complex, Scribble complex (53).

1.4.1 The Par Complex

The Par complex is an evolutionarily conserved functional module including the scaffolding proteins Par-3, Par-6 and atypical protein kinase c (aPKC). Par complex is localized within the apical domain and is responsible for apical membrane determination. Considerable evidence showed that Par complex regulates initial stages of polarization in *C. elegans* (worm) and *Drosophila* (fly) embryos, as well as neuroblast asymmetric cell division in *Drosophila* (54,55). In mammalian epithelial cells, the Par complex has been shown to establish apical–basal polarity and the axon–dendrite polarity of neurons (56). Additionally, it is known to regulate proliferation, asymmetric cell division, and intracellular trafficking. Ultimately the purpose of the Par complex is to control the localization and activation of aPKC, the functional component essential for polarity regulation.

Importantly, three isoforms of aPKC are known to exist in humans: aPKC-ι, aPKC-ζ, and PKM-ζ all of which share a highly conserved kinase domain and aPKC-ι and aPKC-ζ have >70% sequence identity with functional redundancy (54,57). PKM-ζ is a brain-specific isoform and is transcribed from an alternative promoter region within the *PRKCZ* gene (54). Full length aPKC isoforms - aPKC-ι and aPKC-ζ consists of the N-terminal regulatory phem and box-1 (PB-1) domain, the inhibitory pseudosubstrate regulatory sequence (PSR), the catalytic kinase domain and the C1 domain (Fig 1.4). The PB-1 domain of aPKC-ι and aPKC-ζ aids in the binding of aPKCs to the regulatory scaffold proteins Par-6 and p62, which facilitates aPKC-ι and aPKC-ζ localization within the cell, thus regulating their access to the target proteins (58,59). Full length aPKC-ι and aPKC-ζ are held in an autoinhibited state through PSR occupancy of the kinase domain, until phosphorylated by its upstream regulators. The binding of aPKC-ι and aPKC-ζ to Par-6 and p62 induces an allosteric conformational change that displaces the PSR from the kinase domain and allows for kinase activity (60). Since PKM-ζ lacks these key N-terminal regulatory domains (Fig 1.4), it is constitutively active and is thought to play a role in the central nervous system by regulating memory potentiation of post-synaptic endocytosis of key receptors and axon specification in neural stem cell (61).

APKCs are also known to mediate the maintenance of intestinal microvilli by phosphorylating and activating the key microvillar component ezrin (62). Altered expression level and re-localization of aPKCs from the apical domain are associated with human pathologies. APKC-ι mislocalization and elevated expression of aPKCs have been associated with multiple types of cancer (63). Whereas, altered aPKC-ι/ζ localization and decreased expression is associated with irritable bowel syndrome, and microvillar inclusion disease (64–66). Importantly, loss of a differentiated apical surface, defined by reduced abundance of microvilli and altered apical endocytic/exocytic delivery, is an established marker of disturbed epithelial polarity and is also associated with altered aPKC expression levels or localization (67–69). In *Drosophila,* aPKC mutants have been

to exhibit loss of cell polarity with mislocalization of other polarity proteins in neuroblast and epithelial cells (70).

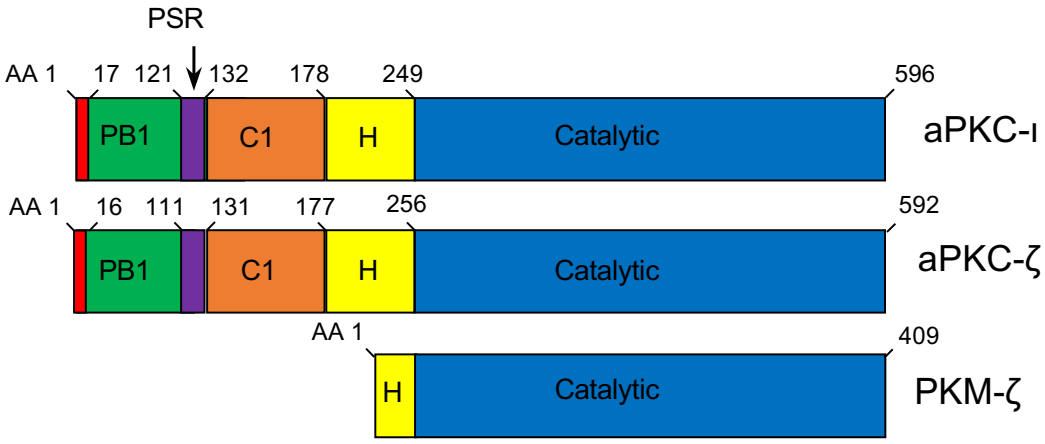


Figure 1.4: Schematic of aPKC isoforms expressed in humans

Full length aPKC isoforms - aPKC-ι and aPKC-ζ consists of the N-terminal regulatory phem and box-1 (PB-1) domain, the inhibitory pseudosubstrate regulatory sequence (PSR), the catalytic kinase domain and the C1 domain. Brain specific aPKC-ζ isoform derived from an alternative promoter region of the *PRKCZ* gene lacks the PB-1 and C1 domain (N-terminally truncated). Modified from Shaha et.al. (2022). Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase c. Placenta.

Par-3 and Par-6 are both adaptor and scaffold proteins containing the PDZ domains. In mice, Par-6 is shown to be indispensable for blastocyst differentiation and establishment of apical basal polarity in the trophoectoderm (71). It has also been shown to negatively regulate syncytialization or the process of fusion of vCTs to form the syncytiotrophoblast in human primary trophoblasts (72). Additionally, it is shown that the apical colocalization of Par-6 with Par-3 in epithelial cells is dependent on the Par-3/aPKC complex activity (59). Despite the interaction between the PDZ domains of Par-3 and the Par-6, phosphorylated Par-3 might lead to its own dissociation from the aPKC-Par-6 complex (59). Since Par-3 is often known to transiently localize near the apical membrane earlier than aPKC and/or Par-6, it is suggested that Par-3 aids in the apical localization

of aPKC-Par-6 complex. Additionally, aPKC kinase activity is shown to be indispensable for the cortical localization of Par-3 (73,74). Taken together, this suggests a dynamic interaction between aPKC and the Par proteins.

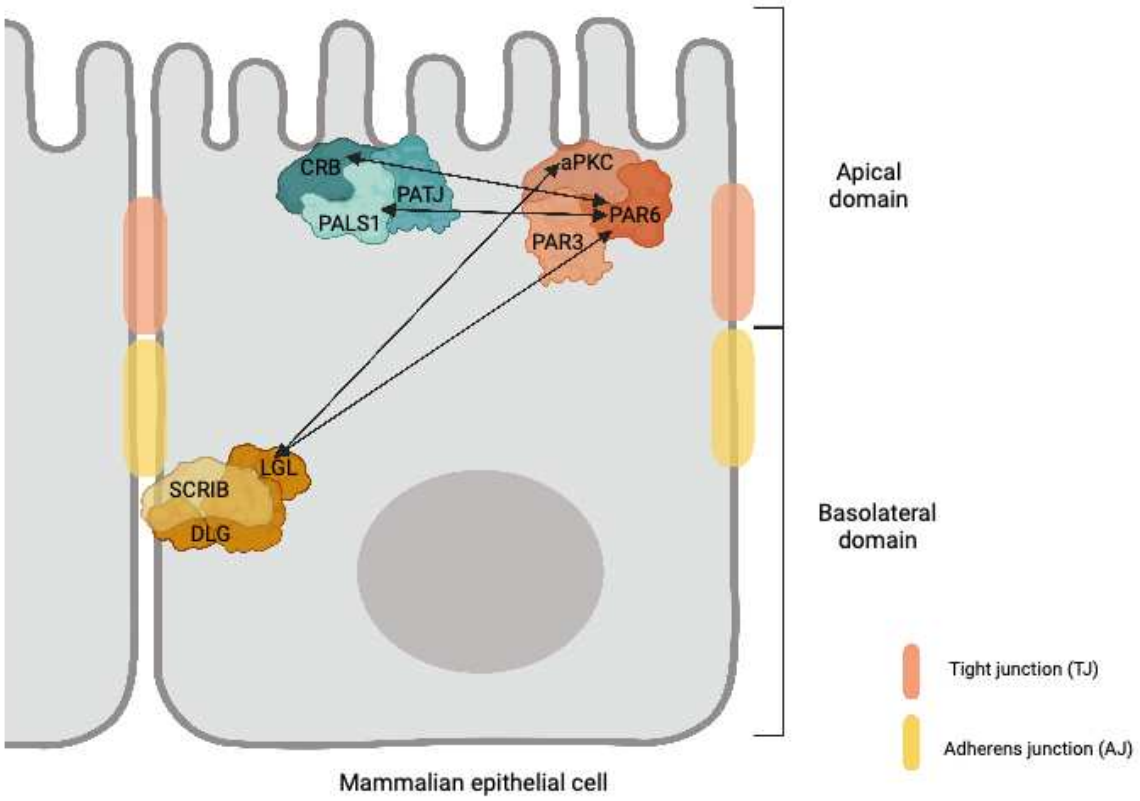
1.4.2. The Crumbs Complex

The Crumbs complex is another apical membrane regulating module consisting of Crumb (Crb), stardust (Sdt; also known as PALS1 in vertebrates) and PALS1-associated tight-junction homologue (PatJ). CRB was originally identified in *Drosophila* as a sub-apical localizing factor responsible for epithelial formation as well as the maintenance of zonula adherence junction in the apical region of *Drosophila* epithelial cells and *CRB* mutants revealed loss of epithelial cell polarity and disorganized epithelium (75–78). Stardust (Sdt) interacts with *CRB* to maintain the zonula adherence junction in *Drosophila* epithelial cells (79). Three human homologues of *Drosophila CRB* (*CRB1*, *CRB2* and *CRB3*) have been identified and characterized as polarity establishment factor in pathological conditions including retinal dystrophy and retinitis pigmentosa (80–82). PALS1, human homologue of *Drosophila* Sdt, was identified as an essential component of crumbs complex during establishment of tight junctions in human cell lines through interaction with human Crb homologues(83,84). PatJ, the human homologue of DpatJ/Disc-lost, was identified in human epithelial cells and shown to interact with CRB3 and to co-localize at tight junction of CaCo2 cells (85). PatJ also plays a key role in directional migration of MDCK cell-mediated through PAR complex (86).

1.4.3. The Scrib Complex

The Scrib complex is a basal determining factor. *SCRIB* was also first identified in *Drosophila* as a regulator of epithelial cell septate junction. *SCRIB* acts along with lethal giant larvae (Lgl) and

disc large (Dlg) (87). Scrib complex establishes basolateral domain in epithelial cells and it functionally interacts with the Par complex in *Drosophila* as well as in mammals during establishment of polarity in epithelial cells (88–92). *SCRIB* complex appears to be important for adherens junction integrity and cell–cell contact (93). In *Drosophila*, Lgl proteins are recruited to the basolateral membrane of the embryonic ectoderm slightly later than the aPKC complex and thereafter play an indispensable role maintaining the apical localization of the aPKC complex (53,55,87). The aPKC complex is essential for restricting Lgl localization to the basolateral membrane (55). The molecular basis of this antagonism is well understood: Lgl competes with Par3 for binding to the aPKC-Par6 complex and thus sequesters the aPKC-Par6 complex away from Par3 (91). In addition, aPKC phosphorylates Lgl to induce a conformational change to an auto-inhibited state in which it interacts with neither the membrane nor the actin cytoskeleton (91). In mammalian epithelial cells, the antagonizing effect of Lgl group proteins on the apical localization of the aPKC complex has not been confirmed.



Created with BioRender.com

Figure 1.5: Schematic of apical-basal determining polarity complexes expressed in mammalian epithelial cells

Mammalian epithelial cells exhibit apical-basolateral polarity that is they have a defined apical domain and basolateral domain. The tight junctions (TJ) in the mammalian epithelial cells are apical to the adherens junctions (AJ). The PAR complex (aPKC-Par3-Par6) is localized apically to TJ, and it interacts with various proteins including the CRUMBS complex (Crb-Pals1-PatJ) that is also localized in the apical domain. APKC belonging to the PAR complex interacts antagonistically with the Lgl protein of the SCRIBBLE complex to maintain apical-basal polarity. Arrows indicate partially known interactions between the complex proteins.

1.4.4 Polarity complex expression and function in the human placenta

Until now, no studies explicitly show the expression and function of Crumbs complex and Scribble complex in the human placenta. However, there have been a few studies involving the

components of Par complex that seem to play a critical role in establishing and maintaining polarity at the apical domain during embryogenesis and syncytialization. Establishment of apical-basal polarity in the embryo is crucial for the segregation of distinct lineages in mammalian development. In mouse embryos, during the late eight-cell stage embryo compaction, polarization is initiated which leads to the formation of apical domain in mouse embryo (94). A recent study, however, showed that aPKC inhibition during early embryonic development leads to impaired trophoectoderm formation implicating a critical role of aPKC in regulating trophoectoderm in early human embryos (95). The trophectoderm layer of the blastocyst eventually forms the placenta and trophoblasts. The ST as mentioned previously is a highly polarized epithelium that performs the key features of the placenta which clearly require active maintenance of polarity. However, how polarity is regulated in this highly specialized cell of the placenta is unknown.

Recently Bhattacharya *et al.* studied the role of aPKC-ı/λ (aPKC-λ in mice) in the development of the murine placenta and the differentiation of TSCs. They found that placental knockout of aPKC-λ in the trophectoderm blocked the development of the labyrinthine exchange zone of the murine placenta. They also found that knockdown of aPKC-ı in human TSC blocked ST differentiation from the TSCs (96). However, they did not examine aPKC-ζ function in human or murine placenta but confirmed that aPKC-ı is expressed in both the ST and CT in humans. PKM-ζ the brain-specific aPKC isoform is transcribed by an internal promoter found within the gene that encodes aPKC-ζ. In a study by Tsokas *et al.*, knock-out of PKM-ζ appeared to be compensated for by activation of aPKC-ı in the mouse hippocampus (56). APKC-λ/ı global knockout mice are embryonic lethal at E9.5 with severe growth restriction while aPKC-ζ global knockout mice have no embryonic phenotype (97). Additionally, it was revealed that aPKC-ζ compensates for aPKC-λ/ı by partially rescuing the phenotypic defects of aPKC-λ/ı knockout mice at E7.5; however, it couldn't prevent embryonic lethality beyond E9.5 (97). Similarly, aPKC-ζ overexpression has been shown to partially compensate for aPKC-ı knockout in mouse embryos (97,98). These suggest the

importance of polarity regulating aPKCs in early murine embryogenesis and the compensatory feature of the aPKCs. These data show that aPKC isoforms may functionally compensate for each other when one is lost or downregulated. Despite sharing 86% kinase domain identity, aPKC-ı and aPKC-ζ seem to have different functions *in vivo*, as aPKC-ζ knockout in mice results in impaired immune function while aPKC-ı knockout is embryonically lethal (98,99). Interestingly, both global and placenta-specific knockout of aPKC-ı is lethal in mouse models, suggesting that aPKCs likely play a critical role in normal placental function and healthy development of the fetus as a result.

Taken together, aPKC seems to play a critical role in the human placenta especially in ST fusion, however it is not yet known if multiple isoforms of aPKC are expressed in the ST. Critically, there is also an evident lack of data that shows how polarity is regulated in ST. Thus, whether this evolutionarily conserved aPKC kinase plays an important role in maintaining ST polarity is unknown.

1.5 Rationale

To sum up, the ST layer of the human placenta plays a critical role in sustaining a healthy pregnancy by aiding in nutrient and gas exchange as well as acting as the immunological barrier and a transient endocrine organ. However, currently there is an evident lack of data addressing this critical feature of the ST. Atypical protein kinase C (aPKC) isoforms have been shown to play a critical role in regulating cell polarity. A recent study has shown the critical role aPKC-ı plays in establishing the maternal fetal interface, however no studies have examined the function of aPKCζ in the human placenta or trophoblasts (94).

Thesis Objectives and Hypothesis

In this thesis, we investigated the role of aPKC isoforms in the regulation of syncytiotrophoblast polarity.

We hypothesize that both isoforms of aPKC are involved in maintaining the ST polarity.

In Chapter 3, we show that the human placenta expresses three isoforms of aPKC – aPKC-ι, aPKC-ζ, and a novel aPKC-ζ isoform - aPKC-ζ III. Our data in Chapter 3 also reveals that aPKC-ι and aPKC-ζ isoforms are expressed at the ST apical membrane in 9-12 week placental tissue and this observation is consistent even in 37-40 week (term) placental tissue. However, due to the lack of antibodies specific for aPKC-ζ III, we were unable to explicitly differentiate between the expression of aPKC-ζ and aPKC-ζ III isoforms. Overall in this chapter, we not only confirm the observations of Bhattacharya *et.al* by showing the presence of aPKC-ι, but also reveal the presence of two aPKC-ζ isoforms in the human placenta. It is the first study to reveal the expression of multiple aPKC isoforms in the human placenta.

In Chapter 4, we show that aPKC isoforms regulate ST polarity. Our data in Chapter 4 reveals the critical role played by aPKC isoforms in mediating ezrin phosphorylation and abundance at the ST apical surface. Importantly, loss of aPKCs led to a significant decrease in ST apical F-actin abundance. We show with isoform specific siRNA knockdown that loss of either of the aPKC isoforms leads to loss of apical F-actin. With our EM images, we show that loss of aPKC kinase activity can lead to decreased ST microvilli with an overall porous ST surface and cytoplasm. We also show that aPKCs regulate ST permeability and that the loss of aPKCs can lead to increased

cellular permeability. Overall, in this chapter, we show that maintenance of ST polarity is critical for regulating ST structural and functional homeostasis.

Chapter 5 of this thesis entails a discussion about the future avenues this study might possibly open to address whether disruption of ST polarity is a cause or consequence in placental pathologies observed in pregnancy complications.

References

1. Shahbazi MN, Zernicka-Goetz M. Deconstructing and reconstructing the mouse and human early embryo. Nat Cell Biol. 2018 Aug;20(8):878–87.

2. Baergen RN, Burton GJ, Kaplan CG. Benirschke's Pathology of the Human Placenta. 2022.

3. Kingdom J, Huppertz B, Seaward G, Kaufmann P. Development of the placental villous tree and its consequences for fetal growth. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2000 Sep;92(1):35–43.

4. Baczyk D, Dunk C, Huppertz B, Maxwell C, Reister F, Giannoulias D, et al. Bi-potential behaviour of cytotrophoblasts in first trimester chorionic villi. Placenta. 2006 May;27(4–5):367–74.

5. Tarrade A, Lai Kuen R, Malassiné A, Tricottet V, Blain P, Vidaud M, et al. Characterization of Human Villous and Extravillous Trophoblasts Isolated from First Trimester Placenta. Lab Invest. 2001 Sep;81(9):1199–211.

6. Lager S, Powell TL. Regulation of Nutrient Transport across the Placenta. Journal of Pregnancy. 2012;2012:1–14.

7. Benirschke K, Burton GJ, Baergen RN. Architecture of Normal Villous Trees. In: Benirschke K, Burton GJ, Baergen RN, editors. Pathology of the Human Placenta [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 101–44. Available from: https://doi.org/10.1007/978-3-642-23941-0_7

8. Boyd JD, Hamilton WJ. The human placenta. Cambridge: Heffer; 1970. 365 p.

9. Huppertz B. The anatomy of the normal placenta. J Clin Pathol. 2008 Dec;61(12):1296–302.

10. Jaremek A, Jeyarajah MJ, Jaju Bhattad G, Renaud SJ. Omics Approaches to Study Formation and Function of Human Placental Syncytiotrophoblast. Front Cell Dev Biol. 2021 Jun 15;9:674162.

11. Burton GJ, Jauniaux E. What is the placenta? Am J Obstet Gynecol. 2015 Oct;213(4 Suppl):S6.e1, S6-8.

12. Simpson RA, Mayhew TM, Barnes PR. From 13 weeks to term, the trophoblast of human placenta grows by the continuous recruitment of new proliferative units: a study of nuclear number using the disector. Placenta. 1992 Oct;13(5):501–12.

13. Mayhew TM, Burton GJ. Stereology and its impact on our understanding of human placental functional morphology. Microsc Res Tech. 1997 Jul 1;38(1–2):195–205.

14. Ishikawa A, Omata W, Ackerman WE, Takeshita T, Vandré DD, Robinson JM. Cell fusion mediates dramatic alterations in the actin cytoskeleton, focal adhesions, and E-cadherin in trophoblastic cells: Trophoblast Fusion and Cellular Alterations. Cytoskeleton. 2014 Apr;71(4):241–56.

15. Renaud SJ, Jeyarajah MJ. How trophoblasts fuse: an in-depth look into placental syncytiotrophoblast formation. Cell Mol Life Sci. 2022 Aug;79(8):433.

16. Brown LM, Lacey HA, Baker PN, Crocker IP. E-cadherin in the assessment of aberrant placental cytotrophoblast turnover in pregnancies complicated by pre-eclampsia. Histochem Cell Biol. 2005 Dec;124(6):499–506.

17. Floridon C. Localization of E-cadherin in villous, extravillous and vascular trophoblasts during intrauterine, ectopic and molar pregnancy. Molecular Human Reproduction. 2000 Oct 1;6(10):943–50.

18. Lu X, Wang R, Zhu C, Wang H, Lin HY, Gu Y, et al. Fine-Tuned and Cell-Cycle-Restricted Expression of Fusogenic Protein Syncytin-2 Maintains Functional Placental Syncytia. Cell Reports. 2017 Oct;21(5):1150–9.

19. Handwerger S. New insights into the regulation of human cytotrophoblast cell differentiation. Mol Cell Endocrinol. 2010 Jul 8;323(1):94–104.

20. Lavialle C, Cornelis G, Dupressoir A, Esnault C, Heidmann O, Vernochet C, et al. Paleovirology of ' *syncytins* ', retroviral *env* genes exapted for a role in placentation. Phil Trans R Soc B. 2013 Sep 19;368(1626):20120507.

21. Teasdale F, Jean-Jacques G. Morphometric evaluation of the microvillous surface enlargement factor in the human placenta from mid-gestation to term. Placenta. 1985 Sep;6(5):375–81.

22. Lash GE, Naruse K, Innes BA, Robson SC, Searle RF, Bulmer JN. Secretion of Angiogenic Growth Factors by Villous Cytotrophoblast and Extravillous Trophoblast in Early Human Pregnancy. Placenta. 2010 Jun;31(6):545–8.

23. Danihel Ľ, Gomolčák P, Korbel M, Pružinec J, Vojtaššák J, Janík P, et al. Expression of proliferation and apoptotic markers in human placenta during pregnancy. Acta Histochemica. 2002 Jan;104(4):335–8.

24. Huppertz B, Gauster M. Trophoblast Fusion. In: Dittmar T, Zänker KS, editors. Cell Fusion in Health and Disease [Internet]. Dordrecht: Springer Netherlands; 2011 [cited 2022 Aug 30]. p. 81–95. (Advances in Experimental Medicine and Biology; vol. 713). Available from: http://link.springer.com/10.1007/978-94-007-0763-4_6

25. Dempsey EW, Luse SA. Regional specializations in the syncytial trophoblast of early human placentas. J Anat. 1971 Apr;108(Pt 3):545–61.

26. McConnell RE, Tyska MJ. Myosin-1a powers the sliding of apical membrane along microvillar actin bundles. Journal of Cell Biology. 2007 May 21;177(4):671–81.

27. McConnell RE, Higginbotham JN, Shifrin DA, Tabb DL, Coffey RJ, Tyska MJ. The enterocyte microvillus is a vesicle-generating organelle. Journal of Cell Biology. 2009 Jun 29;185(7):1285–98.

28. Danielsen EM, Hansen GH. Generation of Stable Lipid Raft Microdomains in the Enterocyte Brush Border by Selective Endocytic Removal of Non-Raft Membrane. Vinciguerra M, editor. PLoS ONE. 2013 Oct 4;8(10):e76661.

29. Sauvanet C, Wayt J, Pelaseyed T, Bretscher A. Structure, Regulation, and Functional Diversity of Microvilli on the Apical Domain of Epithelial Cells. Annu Rev Cell Dev Biol. 2015 Nov 13;31(1):593–621.

30. Viswanatha R, Bretscher A, Garbett D. Dynamics of ezrin and EBP50 in regulating microvilli on the apical aspect of epithelial cells. Biochemical Society Transactions. 2014 Feb 1;42(1):189–94.

31. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine Regulation of Human Fetal Growth: The Role of the Mother, Placenta, and Fetus. Endocrine Reviews. 2006 Apr 1;27(2):141–69.

32. Kidima WB. Syncytiotrophoblast Functions and Fetal Growth Restriction during Placental Malaria: Updates and Implication for Future Interventions. BioMed Research International. 2015;2015:1–9.

33. Azar C, Valentine M, Trausch-Azar J, Druley T, Nelson DM, Schwartz AL. RNA-Seq identifies genes whose proteins are transformative in the differentiation of cytotrophoblast to syncytiotrophoblast, in human primary villous and BeWo trophoblasts. Sci Rep. 2018 Dec;8(1):5142.

34. Tannetta D, Masliukaite I, Vatish M, Redman C, Sargent I. Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia. Journal of Reproductive Immunology. 2017 Feb;119:98–106.

35. Tannetta D, Collett G, Vatish M, Redman C, Sargent I. Syncytiotrophoblast extracellular vesicles – Circulating biopsies reflecting placental health. Placenta. 2017 Apr;52:134–8.

36. Davies R, Griffiths C, Askelund K, Palaiologou E, Cleal JK, Page A, et al. Microvillous tip vesicles may be an origin of placental extracellular vesicles. Placenta. 2022 Jun;123:24–30.

37. Costa MA. The endocrine function of human placenta: an overview. Reproductive BioMedicine Online. 2016 Jan;32(1):14–43.

38. Bloise E, Ortiga-Carvalho TM, Reis FM, Lye SJ, Gibb W, Matthews SG. ATP-binding cassette transporters in reproduction: a new frontier. Hum Reprod Update. 2015 Nov 5;dmv049.

39. Balkovetz DF, Tiruppathi C, Leibach FH, Mahesh VB, Ganapathy V. Evidence for an Imipramine-sensitive Serotonin Transporter in Human Placental Brush-border Membranes. Journal of Biological Chemistry. 1989 Feb;264(4):2195–8.

40. Illsley NP. CURRENT TOPIC: Glucose Transporters in the Human Placenta. Placenta. 2000 Jan;21(1):14–22.

41. Sata R, Ohtani H, Tsujimoto M, Murakami H, Koyabu N, Nakamura T, et al. Functional Analysis of Organic Cation Transporter 3 Expressed in Human Placenta. J Pharmacol Exp Ther. 2005 Nov;315(2):888–95.

42. Cleal JK, Lofthouse EM, Sengers BG, Lewis RM. A systems perspective on placental amino acid transport: A systems perspective on placental amino acid transport. J Physiol. 2018 Dec;596(23):5511–22.

43. Apáti Á, Szebényi K, Erdei Z, Várady G, Orbán TI, Sarkadi B. The importance of drug transporters in human pluripotent stem cells and in early tissue differentiation. Expert Opinion on Drug Metabolism & Toxicology. 2016 Jan 2;12(1):77–92.

44. Karahoda R, Zaugg J, Fuenzalida B, Kallol S, Moser-Haessig R, Staud F, et al. Trophoblast Differentiation Affects Crucial Nutritive Functions of Placental Membrane Transporters. Front Cell Dev Biol. 2022 Feb 22;10:820286.

45. Walker N, Filis P, Soffientini U, Bellingham M, O'Shaughnessy PJ, Fowler PA. Placental transporter localization and expression in the Human: the importance of species, sex, and gestational age differences†. Biology of Reproduction. 2017 Apr 1;96(4):733–42.

46. Delorme-Axford E, Donker RB, Mouillet JF, Chu T, Bayer A, Ouyang Y, et al. Human placental trophoblasts confer viral resistance to recipient cells. Proc Natl Acad Sci USA. 2013 Jul 16;110(29):12048–53.

47. Robbins JR, Skrzypczynska KM, Zeldovich VB, Kapidzic M, Bakardjiev AI. Placental Syncytiotrophoblast Constitutes a Major Barrier to Vertical Transmission of Listeria monocytogenes. Schneider DS, editor. PLoS Pathog. 2010 Jan 22;6(1):e1000732.

48. Robbins JR, Zeldovich VB, Poukchanski A, Boothroyd JC, Bakardjiev AI. Tissue Barriers of the Human Placenta to Infection with Toxoplasma gondii. Adams JH, editor. Infect Immun. 2012 Jan;80(1):418–28.

49. Mengaud J, Ohayon H, Gounon P, Mège RM, Cossart P. E-Cadherin Is the Receptor for Internalin, a Surface Protein Required for Entry of L. monocytogenes into Epithelial Cells. Cell. 1996 Mar;84(6):923–32.

50. Robbins JR, Bakardjiev AI. Pathogens and the placental fortress. Current Opinion in Microbiology. 2012 Feb;15(1):36–43.

51. Bonazzi M, Cossart P. Impenetrable barriers or entry portals? The role of cell–cell adhesion during infection. Journal of Cell Biology. 2011 Oct 31;195(3):349–58.

52. Zeldovich VB, Clausen CH, Bradford E, Fletcher DA, Maltepe E, Robbins JR, et al. Placental Syncytium Forms a Biophysical Barrier against Pathogen Invasion. Wessels MR, editor. PLoS Pathog. 2013 Dec 12;9(12):e1003821.

53. Tanentzapf G, Tepass U. Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. Nat Cell Biol. 2003 Jan;5(1):46–52.

54. Parker SS, Mandell EK, Hapak SM, Maskaykina IY, Kusne Y, Kim JY, et al. Competing molecular interactions of aPKC isoforms regulate neuronal polarity. Proc Natl Acad Sci USA. 2013 Aug 27;110(35):14450–5.

55. Hutterer A, Betschinger J, Petronczki M, Knoblich JA. Sequential Roles of Cdc42, Par-6, aPKC, and Lgl in the Establishment of Epithelial Polarity during Drosophila Embryogenesis. Developmental Cell. 2004 Jun;6(6):845–54.

56. Tsokas P, Hsieh C, Yao Y, Lesburguères E, Wallace EJC, Tcherepanov A, et al. Compensation for PKMζ in long-term potentiation and spatial long-term memory in mutant mice. eLife. 2016 May 17;5:e14846.

57. Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ. Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. J Biol Chem. 1993 Nov 15;268(32):24296–302.

58. Puls A, Schmidt S, Grawe F, Stabel S. Interaction of protein kinase C ζ with ZIP, a novel protein kinase C-binding protein. Proc Natl Acad Sci USA. 1997 Jun 10;94(12):6191–6.

59. Joberty G, Petersen C, Gao L, Macara IG. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol. 2000 Aug;2(8):531–9.

60. Zhang H, Neimanis S, Lopez-Garcia LA, Arencibia JM, Amon S, Stroba A, et al. Molecular Mechanism of Regulation of the Atypical Protein Kinase C by N-terminal Domains and an Allosteric Small Compound. Chemistry & Biology. 2014 Jun;21(6):754–65.

61. Yao Y, Kelly MT, Sajikumar S, Serrano P, Tian D, Bergold PJ, et al. PKM Maintains Late Long-Term Potentiation by N-Ethylmaleimide-Sensitive Factor/GluR2-Dependent Trafficking of Postsynaptic AMPA Receptors. Journal of Neuroscience. 2008 Jul 30;28(31):7820–7.

62. Wald FA, Oriolo AS, Mashukova A, Fregien NL, Langshaw AH, Salas PJI. Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. Journal of Cell Science. 2008 Mar 1;121(5):644–54.

63. Fields AP, Gustafson WC. Protein Kinase C in Disease: Cancer. In: Protein Kinase C Protocols [Internet]. New Jersey: Humana Press; 2003 [cited 2022 Sep 9]. p. 519–38. Available from: http://link.springer.com/10.1385/1-59259-397-6:519

64. Wald FA, Forteza R, Diwadkar-Watkins R, Mashukova A, Duncan R, Abreu MT, et al. Aberrant expression of the polarity complex atypical PKC and non-muscle myosin IIA in active and inactive inflammatory bowel disease. Virchows Arch. 2011 Sep;459(3):331–8.

65. Mashukova A, Wald FA, Salas PJ. Tumor necrosis factor alpha and inflammation disrupt the polarity complex in intestinal epithelial cells by a posttranslational mechanism. Mol Cell Biol. 2011 Feb;31(4):756–65.

66. Kravtsov D, Mashukova A, Forteza R, Rodriguez MM, Ameen NA, Salas PJ. Myosin 5b loss of function leads to defects in polarized signaling: implication for microvillus inclusion disease pathogenesis and treatment. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2014 Nov 15;307(10):G992–1001.

67. Nishimura T, Kaibuchi K. Numb Controls Integrin Endocytosis for Directional Cell Migration with aPKC and PAR-3. Developmental Cell. 2007 Jul;13(1):15–28.

68. Yoshihama Y, Sasaki K, Horikoshi Y, Suzuki A, Ohtsuka T, Hakuno F, et al. KIBRA Suppresses Apical Exocytosis through Inhibition of aPKC Kinase Activity in Epithelial Cells. Current Biology. 2011 Apr;21(8):705–11.

69. Hirate Y, Hirahara S, Inoue K, Kiyonari H, Niwa H, Sasaki H. Par- APKC -dependent and -independent mechanisms cooperatively control cell polarity, Hippo signaling, and cell positioning in 16-cell stage mouse embryos. Develop Growth Differ. 2015 Oct;57(8):544–56.

70. Rolls MM, Albertson R, Shih HP, Lee CY, Doe CQ. Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. Journal of Cell Biology. 2003 Dec 8;163(5):1089–98.

71. Alarcon VB. Cell Polarity Regulator PARD6B Is Essential for Trophectoderm Formation in the Preimplantation Mouse Embryo1. Biology of Reproduction. 2010 Sep 1;83(3):347–58.

72. Sivasubramaniyam T, Garcia J, Tagliaferro A, Melland-Smith M, Chauvin S, Post M, et al. Where Polarity Meets Fusion: Role of Par6 in Trophoblast Differentiation during Placental Development and Preeclampsia. Endocrinology. 2013 Mar 1;154(3):1296–309.

73. Nagai-Tamai Y, Mizuno K, Hirose T, Suzuki A, Ohno S. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells: Regulated interaction between aPKC and PAR-3. Genes to Cells. 2002 Nov;7(11):1161–71.

74. Suzuki A, Ishiyama C, Hashiba K, Shimizu M, Ebnet K, Ohno S. aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. Journal of Cell Science. 2002 Sep 15;115(18):3565–73.

75. Tepass U. Crumbs, a Component of the Apical Membrane, Is Required for Zonula Adherens Formation in Primary Epithelia ofDrosophila. Developmental Biology. 1996 Jul;177(1):217–25.

76. Wodarz A, Grawe F, Knust E. CRUMBS is involved in the control of apical protein targeting during Drosophila epithelial development. Mechanisms of Development. 1993 Dec;44(2–3):175–87.

77. Wodarz A, Hinz U, Engelbert M, Knust E. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of drosophila. Cell. 1995 Jul;82(1):67–76.

78. Klebes A, Knust E. A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in Drosophila. Current Biology. 2000 Jan;10(2):76–85.

79. Bachmann A, Schneider M, Theilenberg E, Grawe F, Knust E. Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. Nature. 2001 Dec 6;414(6864):638–43.

80. Katoh M, Katoh M. Identification and characterization of Crumbs homolog 2 gene at human chromosome 9q33.3. Int J Oncol. 2004 Mar;24(3):743–9.

81. Chartier FJM, Hardy ÉJL, Laprise P. Crumbs controls epithelial integrity by inhibiting Rac1 and PI3K. Journal of Cell Science. 2011 Oct 15;124(20):3393–8.

82. Dudok JJ, Murtaza M, Henrique Alves C, Rashbass P, Wijnholds J. Crumbs 2 prevents cortical abnormalities in mouse dorsal telencephalon. Neuroscience Research. 2016 Jul;108:12–23.

83. Roh MH, Makarova O, Liu CJ, Shin, Lee S, Laurinec S, et al. The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. Journal of Cell Biology. 2002 Apr 1;157(1):161–72.

84. Straight SW, Shin K, Fogg VC, Fan S, Liu CJ, Roh M, et al. Loss of PALS1 expression leads to tight junction and polarity defects. Mol Biol Cell. 2004 Apr;15(4):1981–90.

85. Lemmers C, Médina E, Delgrossi MH, Michel D, Arsanto JP, Le Bivic A. hINADl/PATJ, a Homolog of Discs Lost, Interacts with Crumbs and Localizes to Tight Junctions in Human Epithelial Cells. Journal of Biological Chemistry. 2002 Jul;277(28):25408–15.

86. Shin K, Wang Q, Margolis B. PATJ regulates directional migration of mammalian epithelial cells. EMBO Rep. 2007 Feb;8(2):158–64.

87. Bilder D, Perrimon N. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature. 2000 Feb;403(6770):676–80.

88. Benton R, Johnston DS. Drosophila PAR-1 and 14-3-3 Inhibit Bazooka/PAR-3 to Establish Complementary Cortical Domains in Polarized Cells. Cell. 2003 Dec;115(6):691–704.

89. Yamanaka T. Role of Lgl/Dlg/Scribble in the regulation of epithelial junction, polarity and growth. Front Biosci. 2008;Volume(13):6693.

90. Mu÷sch A, Cohen D, Yeaman C, Nelson WJ, Rodriguez-Boulan E, Brennwald PJ. Mammalian Homolog of Drosophila Tumor Suppressor Lethal (2) Giant Larvae Interacts with Basolateral Exocytic Machinery in Madin-Darby Canine Kidney Cells. Malhotra V, editor. MBoC. 2002 Jan;13(1):158–68.

91. Betschinger J, Mechtler K, Knoblich JA. The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature. 2003 Mar;422(6929):326–30.

92. Betschinger J, Eisenhaber F, Knoblich JA. Phosphorylation-Induced Autoinhibition Regulates the Cytoskeletal Protein Lethal (2) giant larvae. Current Biology. 2005 Feb;15(3):276–82.

93. Laprise P. Emerging Role for Epithelial Polarity Proteins of the Crumbs Family as Potential Tumor Suppressors. Journal of Biomedicine and Biotechnology. 2011;2011:1–9.

94. Chi F, Beniwal AS, Liu H. The apical domain defines the trophectoderm differentiation in early mammalian embryo by regulating YAP nuclear translocation. AME Med J. 2017 Oct;2:155–155.

95. Gerri C, McCarthy A, Alanis-Lobato G, Demtschenko A, Bruneau A, Loubersac S, et al. Initiation of a conserved trophectoderm program in human, cow and mouse embryos. Nature. 2020 Nov 19;587(7834):443–7.

96. Bhattacharya B, Home P, Ganguly A, Ray S, Ghosh A, Islam MdR, et al. Atypical protein kinase C iota (PKCλ/ι) ensures mammalian development by establishing the maternal–fetal exchange interface. Proc Natl Acad Sci USA. 2020 Jun 23;117(25):14280–91.

97. Seidl S, Braun U, Roos N, Li S, Lüdtke THW, Kispert A, et al. Phenotypical Analysis of Atypical PKCs In Vivo Function Display a Compensatory System at Mouse Embryonic Day 7.5. Lim HJ, editor. PLoS ONE. 2013 May 14;8(5):e62756.

98. Soloff RS, Katayama C, Lin MY, Feramisco JR, Hedrick SM. Targeted Deletion of Protein Kinase C λ Reveals a Distribution of Functions between the Two Atypical Protein Kinase C Isoforms. J Immunol. 2004 Sep 1;173(5):3250–60.

99. Martin P. Role of zetaPKC in B-cell signaling and function. The EMBO Journal. 2002 Aug 1;21(15):4049–57.

Chapter 2 – Materials and Methods

2.1 Tissue collection

First trimester (4-12 weeks gestation) and Term (37-40 weeks gestation) human placental samples were collected by methods approved by the University of Alberta human research ethics board. First trimester placental tissue was obtained from elective pregnancy terminations following informed consent from the patients. Term placental tissue was collected from uncomplicated pregnancies after cesarean delivery without labor. Whole mouse brain (3-month old C57BL/6J) collection was approved by the University of Alberta Research Ethics Office in accordance with the Canadian Council for Animal Care Policies.

2.2 Trophoblast isolation and culture

Term or first trimester placental tissue is identified and separated from decidual tissue. Big chunks of placental tissue are cut and a series of 7 enzymatic digestions using Trypsin (Gibco - 27250-018) and DNase is performed (1). The first enzymatic digestion is to get rid of extravillous trophoblasts (EVTs) and the following collections of enzymatic digestion helps to release single cell cytotrophoblasts (1). Following this, tissue is washed in Phosphate Buffer Saline (PBS) [Gibco] and the enzymatically digested tissue is incubated with primary antibodies [anti-CD9 (0.2%v/v); anti-MHC class I (0.16% v/v); anti-MHC class II (0.2% v/v)] to negatively select the cytotrophoblasts as they don't express these proteins. The isolated trophoblasts are then cryopreserved at -80°C until further use.

Isolated cytotrophoblast progenitor cells were seeded into 6 well plates and cultured in Iscove's Modified Dulbecco's Medium (Gibco) + 10% fetal calf serum (Multicell) + penicillin-streptomycin (Gibco) and incubated for 4hrs at 37°C 5%CO2 to obtain in vitro differentiated ST. In order to

remove non-adherent cells, cells were then washed, and medium was changed to IMDM+ 10% FCS + penicillin-streptomycin (5000 IU/mL; Multicell, Wisent Inc.) + 8-bromo-cAMP (10µm, 485 Sigma-Aldrich), and then incubated overnight at 37°C 5%CO2. Then the medium was changed again to remove the 8-bromo-cAMP and the cells were incubated for a further 48hrs 37°C 5% CO2 (cells were in culture for 72hrs in total). Cells were collected after 24 hours (CT) or 72 hours (ST).

2.3 Floating placental explant culture and treatments

Both first trimester and term placental samples were collected and rinsed in cold 1X phosphate-buffered saline (PBS) to remove blood. For first trimester samples, identified placenta was separated from the decidua and cut, trimmed tissue was then washed extensively in PBS to remove blood clots and residual blood. For term placentas, tissue was cut from three central cotyledons, decidua and blood clots were removed, and trimmed tissue was washed extensively in PBS to remove residual blood. Uniform 2 mm^3 explants were then cut from both late first trimester (9-12 weeks) and term (37-40 weeks) tissue and placed in floating culture with appropriate treatments mentioned below.

2.3.1 APKC inhibitor treatment

The uniformly cut explants from both late first trimester (9-12 weeks) and term (37-40 weeks) tissue to be cultured overnight at 37°C 5% CO2 in IMDM (Gibco) supplemented with 10% (v/v) FCS (Multicell, Wisent Inc.) and penicillin streptomycin (5000 IU/mL; Multicell, Wisent Inc.). Explants were washed in serum-free IMDM with 0.1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) following overnight incubation, and incubated for 2-6hrs post adding treatment +/- myristoylated aPKC pseudosubstrate inhibitor (**5 µM**; Invitrogen; Product number 77749) in IMDM

+ 0.1% BSA at 37°C 5% CO_2 (2). Following the treatment, explants were then washed with cold PBS and fixed with 4% paraformaldehyde (prepared fresh daily in 1X PBS) for 2hrs on ice or collected in RIPA lysis buffer for further protein analysis.

2.3.2 PRKCI and PRKCZ targeted siRNA knockdown treatment

Uniformly cut explants from late first trimester placenta (9–12-week) were cultured with IMDM supplemented with 10% (v/v) FCS and gentamicin (50 µg/mL; Thermo Fischer Scientific). siRNA sequences targeting *PRKCI* [final concentration **0.2 nM**; ON-TARGETplus siRNA J-040822-07-0020 (referred as *siRNA-PRKCI#1 in Chapter 4*) ; siRNA J-040822-10-0020 (referred as *siRNA-PRKCI#2 in Chapter 4*); Dharmacon] and *PRCKZ* [final concentration 0.2 nM; ON-TARGETplus siRNA J-003526-17-0010 (referred as *siRNA-PRKCZ#1 in Chapter 4*); siRNA J-003526-14-0010 (referred as *PRKCZ siRNA in Chapter 3; siRNA-PRKCZ#2 in Chapter 4*); Dharmacon], siRNA targeting both *PRKCI* and *PRCKZ (siRNA-PRKCI#1* and *siRNA-PRKCZ#1*), or scrambled control (final concentration **0.2 nM**; ON-TARGETplus Non-targeting Control Pool D-001810-10-20; Dharmacon) were added directly to the medium and incubated for 24hrs at 37°C 5% CO2. Following the knockdown treatment, cold PBS was used to wash the explants before their fixation with 4% paraformaldehyde for 2hrs on ice or collected in RIPA lysis buffer to perform western blotting.

Table 2.1 - Target sequence for siRNA targeting PRKCI and PRKCZ gene

#	**siRNA**	**Target Sequence**
1.	Scramble/Control *(non-targeting)*	UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA
2.	*siRNA-PRKCI#1*	AGAAAUCAGUCUAGCAUUA
3.	*siRNA-PRKCI#2*	GCAAUGAACACCAGGGAAA
4.	*siRNA-PRKCZ#1*	CGUCAAAGCCUCCCAUGUU
5.	*siRNA-PRKCZ#2*	GAGUAUAUCAACCCAUUAU

2.4 Dextran Uptake Assay

During the last 30 minutes of treatment with aPKC inhibitor or siRNA, explants were incubated with 10,000 molecular weight (MW) neutral Dextran Texas Red™ (25 µg/mL; Invitrogen D1828) for 25 minutes suspended in serum-free IMDM and washed with cold 1X phosphate buffered saline (PBS) before fixation with 4% paraformaldehyde for 2hrs on ice.

2.5 Immunohistochemistry and image analysis

Following fixation, whole mount staining was performed for cultured explants or fixed non-cultured placental tissue.

2.5.1 Antigen Retrieval

Antigen retrieval was performed for the primary antibodies that needed better access to the target proteins, namely, anti-ezrin and anti-phospho ezrin. For this, fixed explants were incubated in Tris buffer (pH 7.0) and boiled it in water for 15 mins. Explants were then allowed to cool down to room temperature for 15 mins in distilled water after which they were incubated with blocking buffer [5% normal donkey serum and 0.3% Triton x100, 1% human IgG (Invitrogen) all suspended in PBS] for 2 hrs at room temperature and then tissue staining protocol mentioned below was performed.

2.5.2 Immunofluorescence

For other explants where the primary antibodies did not require antigen retrieval, fixed explants were directly incubated with blocking buffer [5% normal donkey serum and 0.3% Triton x100, 1% human IgG (Invitrogen) all suspended in PBS] for 2 hrs at room temperature.

Then, explants both with or without antigen retrieval were incubated overnight at 4 degrees with primary antibodies (mentioned in Table 2.1) resuspended in a blocking buffer which was diluted 1:1 with PBS. Following this, explants were washed with PBST (0.1% Tween in 1X PBS) and incubated for 2 hours at room temperature with the appropriate secondary antibodies (Alexa Fluor™, Invitrogen) and/or fluorescently conjugated phalloidin (1:400; iFluor 405 or iFluor594; AAT Bioquest) resuspended in a blocking buffer which was diluted 1:1 with PBS. Hoechst 33342 (Invitrogen) was then added for 30 mins. Tissue was then serially washed with PBS-Tween and PBS before being mounted with imaging spacers.

Table 2.2 - List of primary antibodies used for immunohistochemistry (IHC)

#	**Primary Ab**	**Company**	**Catalog #**	**Dilution (µl)**	**Chapter used in**
1.	anti-aPKC-ı	Atlas	HPA025674	1:200	Chapter 3 Chapter 4
2.	anti-aPKC-ζ	Atlas	HPA021851	1:200	Chapter 3 Chapter 4
3.	anti-ezrin	Invitrogen	PA5-18541	1:333	Chapter 4
4.	anti-phospho Thr567 ezrin	Invitrogen	PA5-37763	1:200	Chapter 4
5.	anti β-actin	Cell Signaling Technologies	#8457	1:10000	Chapter 3 Chapter 4
6.	anti-E-cadherin	R&D Systems;	MAB18381	1:200	Chapter 3 Chapter 4
7.	biotinylated-Wheat Germ Agglutinin Lectin	Vector Biolabs		1:200	Chapter 4

2.5.3 Image capture

Images were captured with a Zeiss LSM 700 confocal microscope using a Zeiss 20x/0.8 M27 lens or a Zeiss 63x/1.4 Oil DIC M27 lens. 10-18 µm Z-stack images with a 1 µm step-size were captured at 63x magnification. Three images per treatment were captured. For chapter 4, image capture was restricted to blunt-ended terminal projections of the placental villi from first trimester and term samples.

2.5.4 Image quantitation

All image quantitation was performed with Volocity Imaging software (Version 6.3; Quorum Technologies).

Co-localization analyses- Triplicate regions of interest of the ST apical surface were selected using the freehand tool for each image (three images per treatment), and average global Pearson's correlation coefficient for each region of interest was determined for the anti-aPKC-ι or anti-aPKC-ζ signal and anti-ezrin signal.

Apical Ezrin, Phospho-Ezrin and Phalloidin intensity- Triplicate regions of interest (ROI) were manually selected/drawn using selective cursor in the ST apical surface for each image. Equivalent area between ROIs was ensured by keeping the voxels (pixels in 3-dimension) in a similar range for all ROIs and average mean signal intensity of the phalloidin signal for each ROI was determined.

Dextran uptake- Total villous volume was isolated as a region of interest for each image. Total sum dextran signal was normalized to the total volume of the region of interest. To determine the sum of the dextran signal/volume.

2.6 Electron microscopy

2.6.1 Scanning electron microscopy (SEM)

Following appropriate treatment in culture, triplicate explants from each treatment group were collected and fixed in EM fixative (2.5% Glutaraldehyde: 2% Paraformaldehyde in 0.1 M Phosphate Buffer). For sample preparation, the fixed samples were washed 3 times with 0.1 M Phosphate Buffer, after which the samples were dehydrated through a graded ethanol series. Samples were then serially washed before incubation in a graded series of increasing Hexamethyldisilazane (HMDS) and decreasing ethanol concentration. At 100% HMDS

equilibration, they were washed thrice and left to dry overnight. The samples were then mounted on carbon tape coated stubs, and sputter coated with Au/Pd (Hummer 6.2 Sputter Coater, Anatech). Images were captured with a Zeiss EVO10 SEM microscope using SmartSEM software (Version 6.06).

2.6.2 Transmission electron microscopy (TEM)

Similar to SEM sample preparation, post appropriate treatment, triplicate explants from each treatment group were collected and fixed in EM fixative (2.5% Glutaraldehyde: 2% Paraformaldehyde in 0.1 M Phosphate Buffer). On the day of sample preparation, samples were washed thrice with 0.1 M Phosphate Buffer, then fixed and stained in 1% Osmium tetroxide for 1 hour. Following which these samples were then washed and dehydrated through a graded ethanol series. A 1:1 Ethanol:Spurr Resin mix was prepared in which the dehydrated samples were left overnight. The following day, the samples were incubated with fresh spurr resin, embedded in resin and cured overnight in a 70^{o}C oven. The heat cured sample blocks were then cut in 70-90nm sections using an ultramicrotome (Reichert-Jung Ultracut_E Ultramicrotome). Sections were stained with uranyl acetate and lead citrate on grids and viewed under light microscope to make sure that the cut sections had tissue. Finally, the grids were used to image the samples with a Philips/FEI (Morgagni) TEM, and Digital Micrograph software (Version 1.81.78).

2.7 Western Blotting

RIPA lysis buffer (150mM NaCl,1% Triton-X 100, 0.1% SDS, 50mM Tris, 0.5% Sodium deoxycholate in double distilled water) and protease inhibitor (1:100; Sigma Aldrich; P2714) was added to the collected explants or cells and incubated 10 minutes on ice before sonication and centrifugation at 14000 rpm for 14 minutes. To perform BCA Protein Assay supernatant was

collected post centrifugation. 10-20µg of protein (volume determined via protein assay) was loaded and run on SDS-polyacrylamide gels before transfer onto nitrocellulose membranes. The membranes were then blocked [3% milk powder in TBST (0.1% Tween in 1X Transfer Buffer)] and probed overnight with required primary antibodies resuspended in blocking buffer (See Table 2.2) followed by fluorescent secondary antibodies for detection. Secondary antibodies included Alexa Fluor® donkey anti-mouse 680 (1:10,000; Invitrogen; A28183) and Alexa Fluor® donkey anti-rabbit 800 (1:10,000; Invitrogen; A21039). Following secondary antibody incubation, membranes were washed and visualized using a LI-COR Odyssey Infrared Scanner. For total protein quantification, membranes were first stained using Fast-Green FCF (0.0001% Fast-Green FCF, 30% methanol, 7% acetic acid) for 15 minutes and then de-stained with 30% ethanol and 7% acetic acid before being washed with ddH_2O and scanning on the Licor scanner. Western blot quantitation was performed using the Licor Imaging Software and target protein band intensity was normalized to total protein.

Table 2.3 - List of primary antibodies used for Western Blotting

#	**Primary Ab**	**Company**	**Catalog #**	**Dilution (µl)**	**Chapter used in**
1.	anti-aPKC-ι (mouse)	BD-Biosciences	610207	1:1000	Chapter 3 Chapter 4
2.	anti-aPKC-ζ (rabbit)	Atlas	HPA021851	1:2000	Chapter 3 Chapter 4
3.	anti-total aPKC (mouse)	Santa Cruz	sc-17781	1:1000	Chapter 4
4.	anti β-actin	Cell Signaling Technologies	#8457	1:10000	Chapter 3 Chapter 4

2.8 RNA-isolation and RT-PCR

RNA was isolated using Trizol-chloroform extraction (Life Technologies) and purified with a RNA purification kit (PureLink RNA Mini Kit, Invitrogen). Brain mRNA was isolated using a mRNeasy kit (Qiagen). RNA was reverse transcribed (iScript DNA Synthesis Kit, Biorad) and RT-PCR was performed with SYBR green (Applied Biosystems) on a Quantstudio3 System (Applied Biosystems). ΔCT *PRKCZ* isoforms were calculated to the mean of *TOP1* and *CYC1 (housekeeping genes)* and are displayed as the 2^-(ΔCT).

Table 2.4 - Primer sequences for PRKCZ isoforms

PRKCZ isoform #	**Forward primer sequence**	**Reverse primer sequence**
PRKCZ isoform 1	CCCAAGATGGAAGGGAGCGG	TCCTCACAGAGCTCCTCGAA
PRKCZ isoform 2	TCAACGGGAAGGAAGATGCCT	TCCCCGGCGGTAGATAGATT
PRKCZ isoform 3	GCACGAAAGGGAGAGTTGGA	CCGGCGGTAGATAGATTCCTG
TOP1	GATGAACCTGAAGATGATGGC	TCAGCATCATCCTCATCTCG
CYC1	CAGATAGCCAAGGATGTGTG	CATCATCAACATCTTGAGCC

2.9 ELISA assays

Conditioned medium was collected from technical triplicates of explant culture after 6hrs incubation with treatments and centrifuged at 12,000 rpm for 10 minutes. Post centrifugation, supernatant was aliquoted and stored at -20℃. After washing the explant tissue with PBS, they were flash frozen and stored at -80℃ until total protein was extracted in RIPA lysis buffer post sonication and determined by BCA protein assay, as above. ELISAs were performed using a β-

HCG ELISA kit (DRG International EIA-1911). Plates were read using the Biotek Synergy HTX plate reader (Gen 5 software). The β-HCG values were interpolated using GraphPad PRISM 9 (Version 9.3.1) and normalized to the total protein from the explant the medium was produced by.

2.10 Statistical Analysis

Statistical analyses were completed using GraphPad PRISM 9 (Version 9.3.1.) with an $\alpha = 0.05$ as the threshold for significance. Exact statistical methods used for individual experiments are contained in the figure legends. All graphs represent mean +/- S.E.M.

2.11 References

1. Guilbert LJ, Winkler-Lowen B, Sherburne R, Rote NS, Li H, Morrish DW. Preparation and Functional Characterization of Villous Cytotrophoblasts Free of Syncytial Fragments. Placenta. 2002 Feb;23(2–3):175–83.

2. Bogard AS, Tavalin SJ. Protein Kinase C (PKC) ζ Pseudosubstrate Inhibitor Peptide Promiscuously Binds PKC Family Isoforms and Disrupts Conventional PKC Targeting and Translocation. Mol Pharmacol. 2015 Oct;88(4):728–35.

Chapter 3 – Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c

Authors: Sumaiyah Shaha*, Khushali Patel*, Saba Saadat, Sareh Panahi, Monique M. de Almeida, Anastassia Voronova, Meghan Riddell
* co-first authors
A version of this is published in *Placenta* 2022 *:* https://doi.org/10.1016/j.placenta.2022.01.015

3.1 Introduction

As mentioned in chapter 1, during embryogenesis, the polarized cells become the trophectoderm which adopts an epithelial morphology and eventually forms the placenta(1,2). APKCs are evolutionarily conserved regulators of cell polarity that act as critical functional kinases in establishing and maintaining apical polarity in epithelial cells. They form the Par polarity complex alongside scaffolding proteins Par-6 and Par-3. Importantly, three major isoforms of aPKC exist in humans: aPKC-ι, aPKC-ζ, and PKM-ζ all of which share a highly conserved kinase domain and aPKC-ι and aPKC-ζ have >70% sequence identity with functional redundancy (3). PKM-ζ is specifically expressed in the brain and is transcribed from an alternative promoter region within the *PRKCZ* gene (4).

To re-iterate a section of chapter 1, a study in 2004, showed that aPKC-ζ global knockout mice had no embryonic phenotype whereas aPKC-λ/ι global knockout mice were embryonic lethal at E9 with severe growth restriction (6). Almost a decade later, Seidl et. al revealed that aPKC-ζ can compensate for aPKC-λ/ι$^-$ by partially rescuing the phenotypic defects of aPKC-λ/ι knockout mice at E7.5; however, it couldn't prevent embryonic lethality beyond E9.5 (7). These studies shed light on the importance of polarity regulating aPKCs in early murine embryogenesis and the compensatory feature of the aPKCs, however, no studies further investigated the role of aPKCs in human embryogenesis until much later. Recent studies have established a critical role of

aPKCs in the determination of trophectoderm in human embryogenesis (8). Another study identified one of the aPKC isoforms as a critical regulator of TSC differentiation into the ST. Knockdown of aPKC-ι in human TSC led to marked impairment in the ability of the cells to form ST, the placental side of the maternal-fetal interface (9). Another study found that expression of aPKC isoforms (using an antibody that could not distinguish between aPKC-ι and aPKC-ζ in term placenta was predominantly in the ST microvillus apical membrane, though signal could also be detected in ST basal and apical membrane preparations by western blotting in tissue from 16 weeks gestation to term (10). These observations are in sync with the previous literature on aPKC that showed a strong apical localization of aPKC isoforms in epithelial cells as most of the aPKC substrates are apical membrane-bound proteins.

Thus, aPKCs play a critical role in the determination of trophectoderm early in gestation. The absence of aPKC-ι leads to altered ST formation in human TSCs and compensatory activation of remaining isoforms when individual aPKC isoform expression is lost has been observed. However, there were no studies investigating the protein expression of aPKC-ζ in the human placenta or trophoblasts.

3.2 Objective

Hence, we wanted to examine which isoforms of aPKC are expressed in early and late gestation human placenta and trophoblasts to begin to address their function in definitive human trophoblasts.

3.3 Results

To confirm the expression of aPKC-ι in the human placenta as observed by Bhattacharya *et.al*, we performed aPKC-ι indirect immunofluorescence in human first trimester placental tissue (For methods see section 2.5). We observed a strong anti-aPKC-ι signal in the proliferative vCTs which was highly co-localized with E-cadherin (CT marker adherens-junction protein) whereas a largely diffuse anti-aPKC-ι signal in the ST of the early first trimester (4-7 weeks) placental villi (Fig 3.1A). However, we identified a few areas in the ST apical membrane with a strong anti-aPKC-ι signal, but this was largely inconsistent amongst different villi of the same placental sample. Importantly, a similar staining pattern in the vCTs with a weak nuclear pattern but a strong apical anti-aPKC-ι signal in the ST of the late first trimester placental villi (9-12 weeks) was observed (Fig 3.1B). In term placenta (37-40 weeks), the aPKC-ι signal remained strong in the vCT, though the signal could be observed throughout the cell, including in the nucleus (Fig 3.1C). However, in contrast to Bhattacharya *et al*. a stronger ST signal that was accumulated at the apical membrane was observed.

An antibody targeting aPKC-ζ isoforms revealed similar staining patterns to aPKC-ι. In the early first trimester, the strongest signal was observed in the vCT which was highly co-localized with E-cadherin (CT marker-junctional protein) and a largely diffuse signal was observed in the ST. Inconsistent patches of apical accumulation of signal in the ST were observed starting at 6 weeks gestation (Fig 3.2A). In contrast to aPKC-ι, no consistent anti-aPKC-ζ signal overlapped with the nuclear stain in any trophoblast population. In the late first trimester samples (9-12 weeks), the anti-aPKC-ζ signal in the vCT co-localized with E-cadherin and a strong ST signal was also observed (Fig 3.2B). There was a persistent ST apical localization of the aPKC-ζ at this point in gestation, though a cytoplasmic signal was also evident. The staining pattern of aPKC-ζ in term tissue again resembled that of aPKC-ι, with a strong signal observed in the vCT

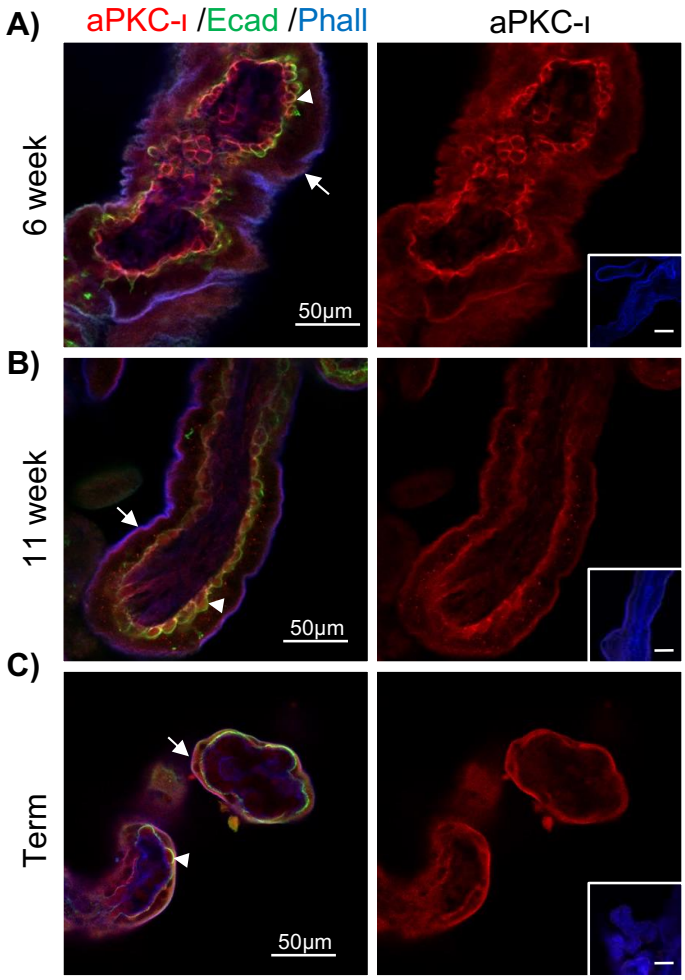


Figure 3.1: Human placental trophoblasts in early and late gestation express aPKC-ι isoform.

Signal for aPKC-ι (red), E-cadherin (green) and phalloidin (blue) in A) 6-week, B) 11-week, and C) term placenta (left panels) and aPKC-ι only (right panels); inset image negative control; Arrows indicate location of ST apical membrane; arrowheads= vCT; All images are representative of a minimum of n=3 samples collected from separate patients; scale bars= 50µm; all images presented in a single focal plane. (11)[1]

[1] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.1A). Published in *Placenta*. 2022

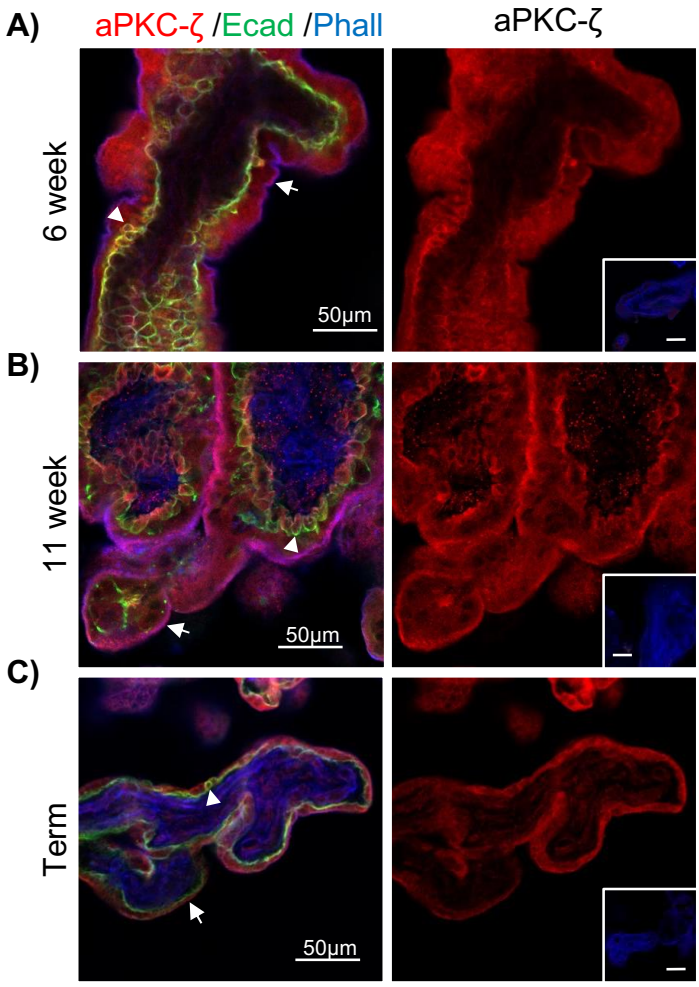


Figure 3.2: Human placental trophoblasts in early and late gestation express aPKC-ζ isoform.

Signal for aPKC-ζ (red), E-cadherin (green) and phalloidin (blue) in A) 6-week, B) 11-week, and C) term placenta (left panels) and aPKC-ζ only (right panels); inset image: negative control; Arrows indicate location of ST apical membrane; arrowheads= vCT; All images are representative of a minimum of *n*=3 samples collected from separate patients; scale bars= 50µm; all images presented in a single focal plane.(11)[2]

co-localized with E cadherin. Like the late-first trimester ST (9-12 weeks), the aPKC-ζ signal was accumulated at the term (37-40 weeks) ST apical membrane but was also observed throughout the cytoplasm (Fig 3.2C). Thus, aPKC isoforms are predominantly expressed in the trophoblast

[2] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.1C). Published in *Placenta*. 2022

lineage at all gestational ages examined and have largely similar localization within trophoblastic cells. To confirm our observations, we performed western blotting on whole placental lysate, primary cultured CT, and *in vitro* differentiated ST (For methods see section 2.7). Using an aPKC-ι specific antibody, expression was observed in whole placental lysate at all gestational ages examined and was stronger though not statistically significant in primary isolated CT than ST, mirroring the pattern observed by immunofluorescence. Surprisingly, two prominent bands were consistently present when using aPKC-ζ specific and an antibody that recognizes all aPKC isoforms (total aPKC). As expected, a ~70 kDa band which correlates to the molecular weight of aPKC-ζ was observed, however, a strong ~55 kDa band was also observed (Fig 3.3). PKM-ζ, the brain-specific *PRKCZ* isoform, has an approximate molecular weight of 50 kDa, thus to confirm if PKM-ζ is expressed in human placenta and isolated trophoblasts, whole placental lysate samples were run alongside whole mouse brain lysates to see if the placental and trophoblast ~55 kDa band matched the molecular weight of PKM-ζ. Surprisingly, the ~55 kDa band in all placental and trophoblast samples was observed to run slightly higher than the molecular weight of PKM ζ observed in the brain. We also performed *PRKCZ* targeted siRNA knockdown on first trimester placental explants to confirm the specificity of the antibody that stained for aPKC-ζ (Fig 3.4). Western blot analysis of these knockdown samples revealed a significant decrease in both the 70 kDa and 55 kDa bands.

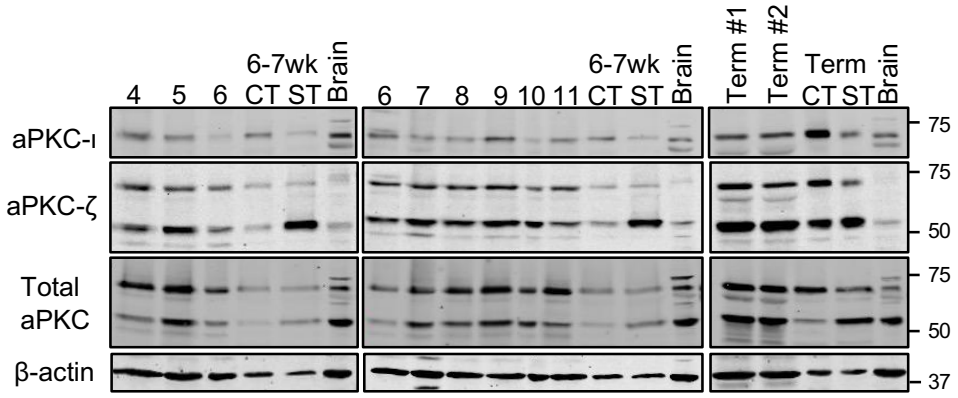


Figure 3.3: Western blotting of first and third trimester placenta and isolated primary cultured CT and ST show a 55kDa and 75kDa band with anti-aPKC-ζ targeting antibodies.

Western blotting analyses with anti-aPKC-ι, anti-aPKC-ζ, anti-total aPKC, and anti-β-actin for 4-11 week and term whole placental lysates, cultured CT and ST from cells isolated from 6-7 weeks gestation and term placentas, and mouse brain. Representative of *n*≥ 3 placentas and cell isolations.(11)[3]

[3] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.2A). Published in *Placenta*. 2022

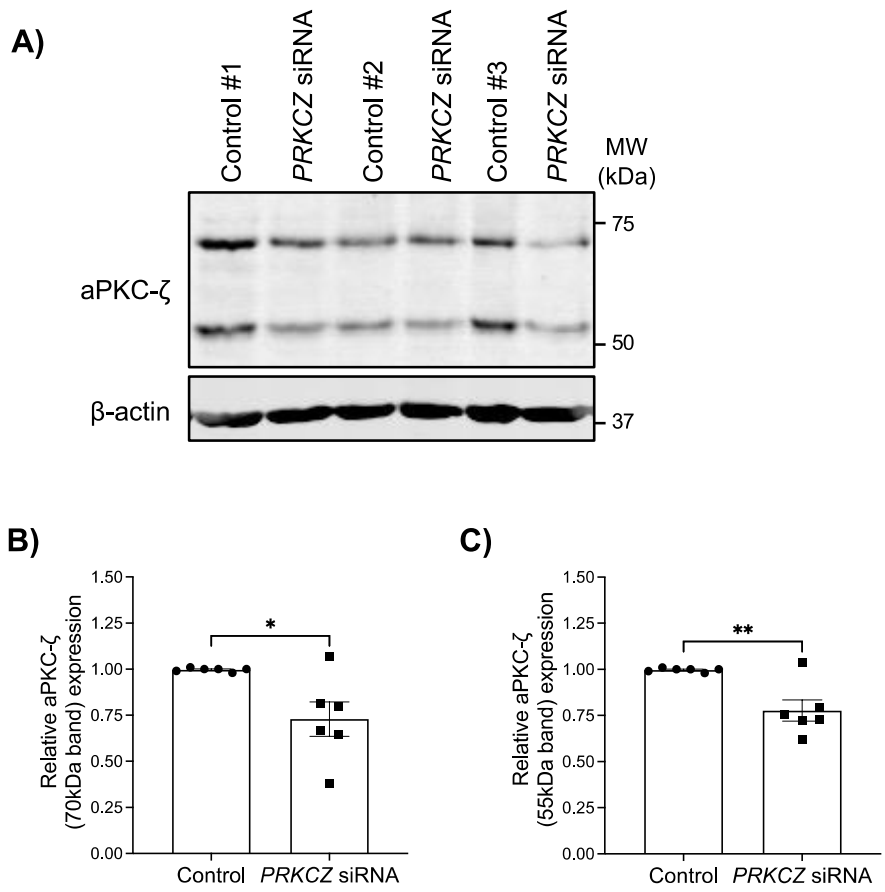


Figure 3.4: siRNA KD validation of anti-aPKC-ζ antibody.

A) Representative western blotting analysis with anti-aPKC-ζ and anti-β-actin of control and *PRKCZ* targeting siRNA treated first trimester placental explant lysate where n=3; Western blot quantification for B) 70kDa aPKC isoform and C) ~55kDa aPKC isoform protein expression from siRNA treated explants; mean+/- S.E.M; unpaired t-test; *=p<0.05; **=p<0.01; *n*=6 (11)[4]

Despite the confirmed expression of only aPKC-ζ and PKM-ζ, multiple different mRNA products and their predicted proteins have been annotated for the *PRKCZ* gene. To examine which *PRKCZ* mRNA isoforms may be expressed in the placenta and trophoblasts, primers were designed so they were specific to the full-length *PRKCZ* mRNA isoform 1 (full-length aPKC-ζ), as well as

[4] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.2B-C). Published in *Placenta*. 2022

PRKCZ mRNA isoform 2 (brain-specific PKM-ζ) and *PRKCZ* mRNA isoform 3, which would encode a protein that lacks the N-terminal PB-1 domain and has a predicted molecular weight of 56 kDa (Table 2.3). Using RT-PCR, the primers targeting *PRKCZ* isoform 1 successfully amplified product in first and third trimester whole placental lysate as well as primary cultured CT and *in vitro* differentiated ST (Fig 3.5). *PRKCZ* isoform 2 specific primers successfully amplified product from human brain lysate, but not from placental lysate or isolated trophoblasts (Fig 3.6). Interestingly, primers targeting *PRKCZ* isoform 3 were able to amplify products from the human placenta and trophoblasts. Thus, mRNA encoding two aPKC isoforms, *PRKCZ* isoform 1 and the N-terminally truncated PRKCZ isoform 3, are present in human placenta and trophoblasts (Fig 3.7). Importantly, these mRNA products would produce proteins with the same molecular weights as those detected by western blot analysis.

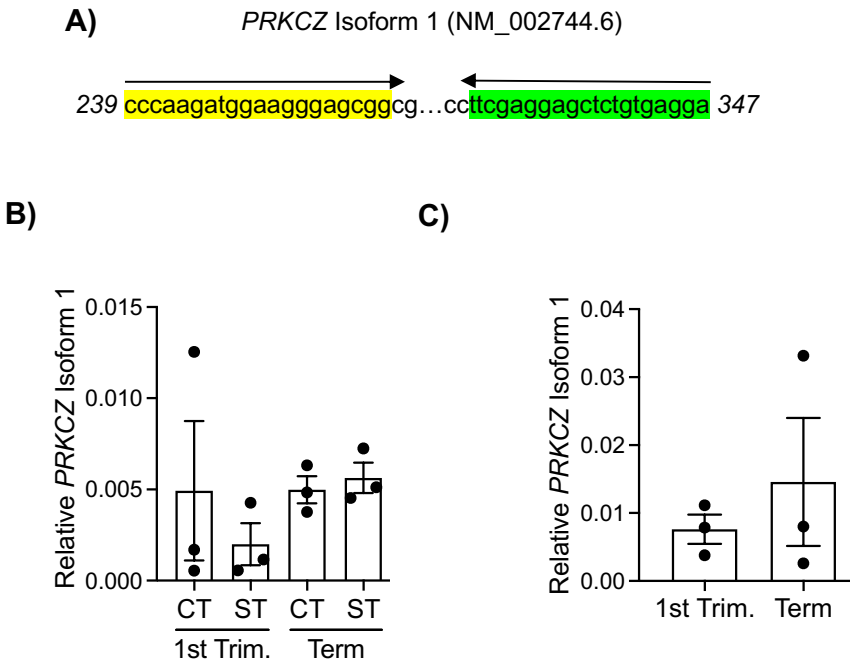


Figure 3.5: PRKCZ isoform specific primers amplify isoform 1 mRNA in villous trophoblasts and whole placental lysate.

A) *PRCKZ* isoform 1 (full length aPKC-ζ) specific primer binding sites (highlighted sequences) B) Relative expression in first trimester (9-12 week) and term (37-40 week) CT, and ST and C) Relative expression in placental lysate; All data presented as 2^-ΔCT to *TOP1* and *CYC1*; *n*=3; mean+/- S.E.M (11)[5]

[5] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.2D-F). Published in *Placenta*. 2022

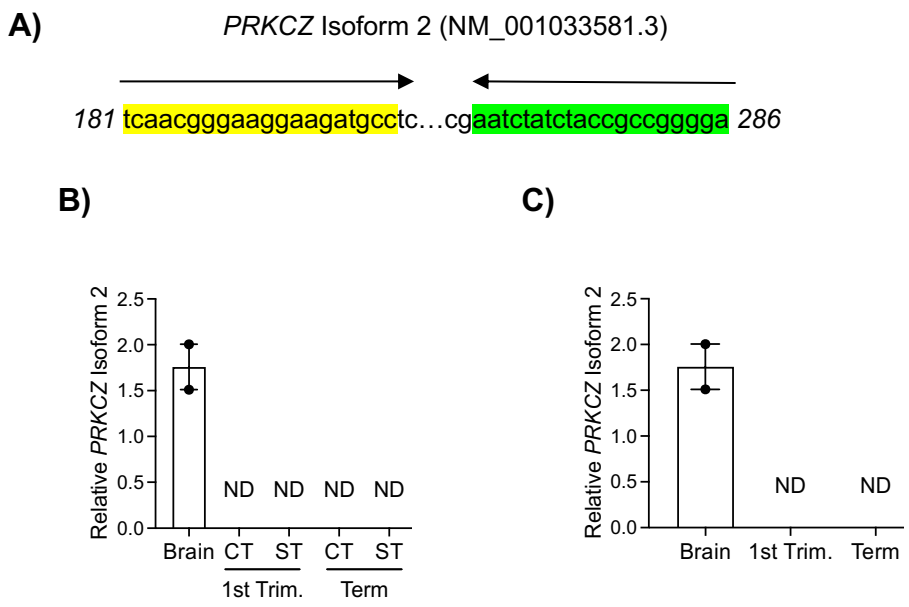


Figure 3.6: PRKCZ isoform specific primers amplify isoform 2 mRNA only in the human brain.

A) *PRKCZ* isoform 2 (PKM-ζ) specific primer binding sites (highlighted sequences). B) Relative expression in first trimester (9-12 week) and term (37-40 week) CT and ST and C) Relative expression in placental and human brain lysate; All data presented as 2^-ΔCT to *TOP1* and *CYC1*; *n*=3 for all except the brain where *n=2*; mean+/- S.E.M (11)[6]

[6] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.1G-I). Published in *Placenta*. 2022

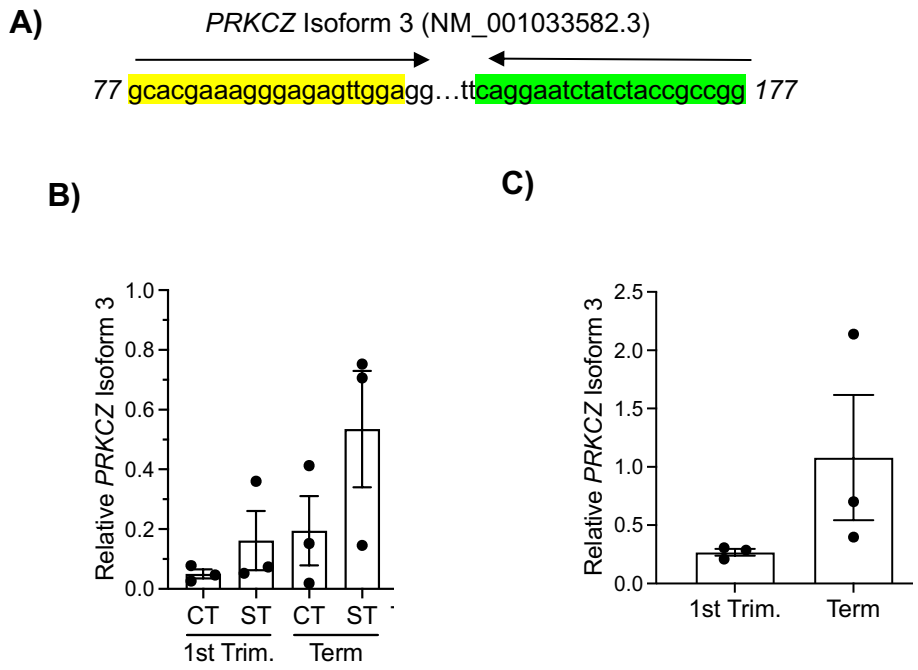


Figure 3.7: PRKCZ isoform specific primers amplify isoform 3 mRNA in villous trophoblasts and whole placental lysates.

A) *PRKCZ* isoform 3 specific primer binding sites (highlighted sequences) and B) Relative expression in first trimester (9-12 week) and term (37-40 week) CT, and ST and C) Relative expression in placental lysate; All data presented as 2^-ΔCT to *TOP1* and *CYC1*; *n*=3; mean+/- S.E.M(11)[7]

3.4 Discussion

We have found that human placental trophoblasts simultaneously express three isoforms of aPKC. aPKC-ι was more often found within the nuclear compartment of all trophoblastic populations than aPKC-ζ, suggesting it may play a distinct role from aPKC-ζ in the nucleus. But, importantly, the high degree of apparent overlap in the localization and expression of both aPKC-ι and aPKC-ζ throughout gestation suggests considerable functional redundancy could be possible between the isoforms. Hence, the possibility for compensatory activation and re-

[7] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.2J-M). Published in *Placenta*. 2022

localization of aPKC-ζ in aPKC-ι KD conditions must be thoroughly addressed.

Full-length aPKC isoforms require interaction with scaffolding proteins, such as Par-6, Par-3, or p62, to fully activate and regulate their localization. The temporal and spatial regulation of full-length aPKC isoform activation by its binding partners is a critical regulatory mechanism ensuring access to specific targets depending on the cellular environment (3,12). These interactions are required for the regulation of apical-basal polarity. PRKCZ isoform 3 (aPKC-ζ III) mRNA sequence does not encode the N-terminal PB-1 domain through which aPKC-ι/ζ interacts with Par-6 and p62 (Fig. 3.8). Importantly, both aPKC-ι and aPKC-ζ interact with Par-3, which in certain contexts is also a phosphorylation target of aPKC's, through a conserved region of their kinase domain (12). Importantly, PKM-ζ has been shown to interact with Par-3 and compete with aPKC-ι/λ (aPKC-λ in mice) for interaction with Par-3 to allow for axon polarization in neurons (5).

Therefore, similar to PKM-ζ, with its N terminally-truncated structure, aPKC-ζ III is likely able to interact with Par-3 and could play a similar role in regulating the occupancy of aPKC-ι and aPKC-ζ within the Par complex in trophoblasts. If occurring, this would represent a unique regulatory mechanism governing a critical polarity regulatory complex. Co-localization of total aPKC with Par-6 and Par-3 was shown in the trophectoderm of human and murine blastocysts, suggesting that aPKC isoforms may be integrated into the Par complex, in line with their function in establishing polarity at this stage of development (8,13). Whether aPKC-ζ III is also expressed in the preimplantation and early post-implantation trophectoderm remains to be established. But since all studies that have ascribed a function for aPKC in the early human trophectoderm, used methods that would target all three isoforms elucidating the roles for the individual types of aPKC is required.

Presently, the role of aPKC-ζ III in trophoblasts is unknown. aPKC-ζ III protein expression was found to be highest in the strongly polarized ST, where aPKC-ι expression is lowest and aPKC- ζ is also expressed (Fig 3.3). Similar localization could be observed with both aPKC-ι and aPKC-ζ/

aPKC-ζ III specific antibodies, therefore competition for common binding partners is likely to occur.

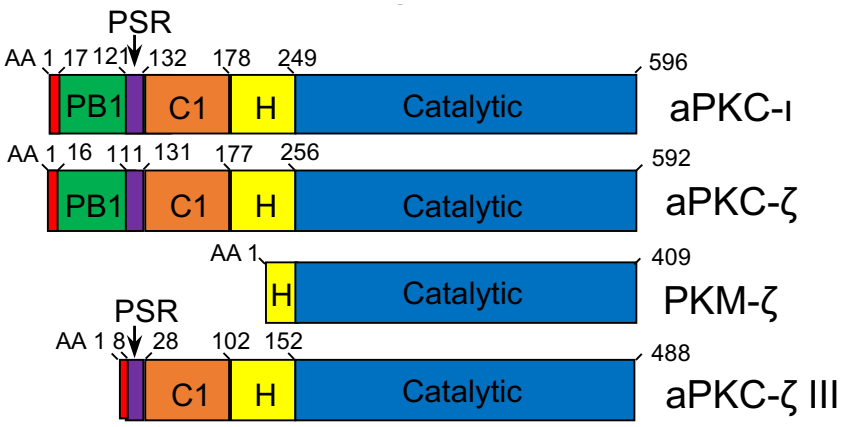


Figure 3.8: Schematic representation of aPKC isoforms in humans

Three isoforms of aPKC are expressed in the first and third trimester placenta and isolated CT and ST; Representative domain structure of human aPKC-isoforms; PSR= pseudosubstrate regulatory region; H= hinge; PB1 = Phem Box 1(11)[8].

However, there may be compartmentalization of aPKC-ζ and aPKC ζ III within the ST to allow them to carry out independent functions due to differing affinity for adaptor/scaffolding partner interactions by the different isoforms. Presently the exact cellular distribution of these individual isoforms is unclear as the antibody used for staining in this study cannot distinguish the signal from aPKC-ζ and aPKC-ζ III. Future studies to identify the binding partners of aPKC-ι, aPKC-ζ, and aPKC-ζ III in the trophoblasts will help to illuminate potential roles for the individual isoforms

[8] Modified from Shaha *et.al.* Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c (Fig.2M). Published in *Placenta*. 2022

in maintaining ST polarity and allow for a better understanding of their localization.

Finally, it is presently unclear whether aPKC-ζ III is capable of full activation and if it possesses a kinase activity similar to full-length aPKC-ζ. *In vitro* expression of an aPKC-ζ deletion mutant lacking the first 98 amino acids, therefore 5 amino acids shorter than aPKC-ζ III, was found to be lipid activatable, like full-length aPKC-ζ and aPKC-ι, and possessed a similar kinase activity in the presence of lipids to full-length aPKC-ζ (14). Since aPKC-ζ III contains both the C1 domain and PSR that allow for autoinhibition but lacks the PB-1 domain through which binding of full-length aPKC isoforms leads to allosteric conformational changes and activation, the regulation of aPKC-ζ III is likely, at least partially, distinct from aPKC-ζ and aPKC-ι.

In conclusion, our data identifies the unique sustained simultaneous expression of three isoforms of the aPKC family in human trophoblasts. Even though it is not possible to differentiate between aPKC-ζ /aPKC-ζ III localization, we now know that aPKC-ι and aPKC-ζ isoforms localize apically in the ST. With previous literature showing a pivotal role for aPKCs in trophectoderm and trophoblast lineage-specific differentiation, identifying the function and regulation of individual aPKCs in the highly polarized ST epithelium will be an important direction for understanding the molecular regulation of polarity in the ST lining the human placenta at the maternal-fetal interface.

3.5 References

1. Shahbazi MN, Zernicka-Goetz M. Deconstructing and reconstructing the mouse and human early embryo. Nat Cell Biol. 2018 Aug;20(8):878–87.

2. Marikawa Y, Alarcon VB. Creation of Trophectoderm, the First Epithelium, in Mouse Preimplantation Development. In: Kubiak JZ, editor. Mouse Development [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012 [cited 2022 Aug 14]. p. 165–84. (Results and Problems in Cell Differentiation; vol. 55). Available from: http://link.springer.com/10.1007/978-3-642-30406-4_9

3. Hong Y. aPKC: the Kinase that Phosphorylates Cell Polarity. F1000Research. 2018 Jun 25;7:903.

4. Yao Y, Kelly MT, Sajikumar S, Serrano P, Tian D, Bergold PJ, et al. PKM Maintains Late Long-Term Potentiation by N-Ethylmaleimide-Sensitive Factor/GluR2-Dependent Trafficking of Postsynaptic AMPA Receptors. J Neurosci. 2008 Jul 30;28(31):7820–7.

5. Parker SS, Mandell EK, Hapak SM, Maskaykina IY, Kusne Y, Kim JY, et al. Competing molecular interactions of aPKC isoforms regulate neuronal polarity. Proc Natl Acad Sci. 2013 Aug 27;110(35):14450–5.

6. Soloff RS, Katayama C, Lin MY, Feramisco JR, Hedrick SM. Targeted Deletion of Protein Kinase C λ Reveals a Distribution of Functions between the Two Atypical Protein Kinase C Isoforms. J Immunol. 2004 Sep 1;173(5):3250–60.

7. Seidl S, Braun U, Roos N, Li S, Lüdtke THW, Kispert A, et al. Phenotypical Analysis of Atypical PKCs In Vivo Function Display a Compensatory System at Mouse Embryonic Day 7.5. Lim HJ, editor. PLoS ONE. 2013 May 14;8(5):e62756.

8. Gerri C, McCarthy A, Alanis-Lobato G, Demtschenko A, Bruneau A, Loubersac S, et al. Initiation of a conserved trophectoderm program in human, cow and mouse embryos. Nature. 2020 Nov 19;587(7834):443–7.

9. Bhattacharya B, Home P, Ganguly A, Ray S, Ghosh A, Islam MdR, et al. Atypical protein kinase C iota (PKCλ/ι) ensures mammalian development by establishing the maternal–fetal exchange interface. Proc Natl Acad Sci. 2020 Jun 23;117(25):14280–91.

10. Visco I, Hoege C, Hyman AA, Schwille P. In vitro Reconstitution of a Membrane Switch Mechanism for the Polarity Protein LGL. J Mol Biol. 2016 Dec;428(24):4828–42.

11. Shaha S, Patel K, Saadat S, Panahi S, de Almeida MM, Voronova A, et al. Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c. Placenta. 2022 Mar;119:39–43.

12. Nagai-Tamai Y, Mizuno K, Hirose T, Suzuki A, Ohno S. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells: Regulated interaction between aPKC and PAR-3. Genes Cells. 2002 Nov;7(11):1161–71.

13. Plusa B, Piliszek A, Frankenberg S, Artus J, Hadjantonakis AK. Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. Development. 2008 Sep 15;135(18):3081–91.

14. Lopez-Garcia LA, Schulze JO, Fröhner W, Zhang H, Süß E, Weber N, et al. Allosteric Regulation of Protein Kinase PKCζ by the N-Terminal C1 Domain and Small Compounds to the PIF-Pocket. Chem Biol. 2011 Nov;18(11):1463–73.

Chapter 4 – APKC isoforms maintain ST apical surface

Authors: Khushali Patel*, Jasmine Nguyen*, Sumaiyah Shaha, Ashley Zubkowski, Meghan Riddell
* co-first authors
A version of this is published as a pre-print in *Biorxiv* 2022: https://doi.org/10.1101/2022.06.30.498172

4.1 Introduction

As presented in Chapter 3, three isoforms of aPKC are expressed in the highly polarized ST: aPKC-ι, aPKC-ζ, and a novel N-terminal truncated *PRKCZ* encoded isoform – aPKC-ζ III (1). Importantly, aPKC isoforms have both redundant and exclusive functions. As previously mentioned in Chapter 1, the human placenta is lined by the ST at its maternal-fetal interface. The ST is a unique giant multinucleate cell formed by the fusion of underlying proliferative vCTs. This ST is further differentiated apically to form membrane projections, called microvilli, which are bathed in the maternal blood.

Microvilli are a hallmark of established apical polarity in epithelial cells and are formed and maintained by active F-actin reorganization. As introduced in section 1.3.3.1, microvilli are supported by an F-actin core which anchors to the microvillar membrane via cytoskeletal linker proteins from the ERM family. Critically ezrin, links the microvillar F-actin core to the cell membrane only when activated via phosphorylation at Thr567 site. and is expressed abundantly at the ST apical surface at the end of gestation (2,3). Phosphorylation at Thr567 in ezrin activates the typically dormant configuration of ezrin such that the C-terminal domain of ezrin binds to the F-actin and the N-terminal domain binds to the microvillar membrane linking the F-actin core to the membrane and thus maintaining structural integrity. Alterations in ezrin levels have been linked to various pathologies such as Alzheimers, asthma, cancer metastasis and retinal microvilli loss and disruption (4). Loss of ezrin has also been shown to perturb the established F-actin cytoskeleton in Sertoli cells (5). Nishimura *et. al* show that *Ezr* knockout mice have fetal growth

retardation and that ezrin is required for uptake of critical fetal growth factors from maternal serum by placental trophoblasts (6). These data reveal the importance of ezrin expression and its phosphorylation in various cell systems. Importantly, ezrin is known to express abundantly at the ST apical surface (2). Thus, active ezrin can be used as an apical marker as a readout for established polarity.

Several kinases have been shown to phosphorylate ezrin at Thr567 apically in epithelial cells including Mst4 in intestinal cell line (7), PKCα in migrating cells, and PKCθ in human skin carcinoma cell line (8,9). Akt2 has also been shown to phosphorylate ezrin at Thr567 in intestinal cell lines (10,11). APKC-ι/λ isoform has been shown to regulate the emergence and maintenance of intestinal microvilli by phosphorylating ezrin at Thr567 in CACO-2 cell line (13). Importantly, Pearson and Kemp showed that the Thr567 is localized within a highly conserved PKC consensus phosphorylation site (14). Despite the known apical expression of ezrin in the placental trophoblasts, there is only one study that has looked at ezrin phosphorylation in human trophoblastic cells. Miura *et. al* showed that Akt inhibition in the BeWo choriocarcinoma cell line, led to decreased microvilli formation in these transformed cells (16). However, no studies have examined the regulation of ezrin and its phosphorylation in a non-transformed human placental cell model.

As mentioned above, apical emergence of microvilli is a hallmark of established cell polarity. Microvilli are actin rich membrane protrusions, which are actively maintained by dynamic actin reorganization. F-actin is known to play a critical role in various cellular functions like maintaining cell structure, cell motility, cell signaling, vesicular trafficking as well as endocytosis (17). Alterations in F-actin in different systems have been linked with disturbed vesicular trafficking, loss of dendritic spines leading to Alzheimer's as well as cell death (18–20). Importantly, the human ST has a dense network of actin-supported microvilli where vesicular trafficking, endocytosis and various other functions maybe directly or indirectly regulated via actin dynamics

(17,18,20). However, aPKCs are also known to regulate actin dynamics via multiple pathways (21). But no studies have examined the role of polarity complexes in the ST.

Presence of microvilli is indicative of established polarity. Now we also know that multiple aPKC isoforms are expressed in the ST and aPKC-ι is shown to regulate ezrin phosphorylation in intestinal microvilli as well as trophoblastic fusion (13,16). Thus, it would be critical to understand whether these multiple aPKC isoforms regulate ST polarity exclusively or redundantly. However there has been a huge gap in the literature in understanding whether ST polarity and its associated functions are regulated via aPKC isoforms. Ezrin localization and activation along with presence of actin rich microvilli can therefore be used as tools to determine the presence of ST apical polarity.

4.2 Hypothesis

Thus, we hypothesized that aPKC isoforms expressed in the human ST regulate ST polarity and apical ezrin expression.

4.3 Results

As shown in Chapter 3, our initial studies investigating aPKC isoforms in the human placenta revealed a weak anti-aPKC-ι or anti-aPKC-ζ/ζ III signal accumulation at the ST apical membrane in the early first trimester (4-6 weeks) placental samples. Critically, we observed a strong apical accumulation of aPKC isoforms during the late first trimester (9-12 weeks). In other cell types, aPKC is required to localize apically and phosphorylate ezrin to regulate apical-basal polarity and establish apical microvilli as mentioned above (13). So, a colocalization analysis was performed

using antibodies targeting aPKC-ı or aPKC-ζ isoforms (that we previously validated to recognize aPKC-ζ and aPKC-ζ III (See Chapter 2, Figure 3.1-3.2; (1)) and an anti-ezrin antibody (For methods see section 2.5). The anti-aPKC-ı signal in the ST was sparse and largely diffuse in the early first-trimester placenta where we also observed an inconsistent accumulation of anti-ezrin signal at the ST apical surface and relatively weak colocalization coefficients were quantified (Fig 4.1). Additionally, the anti-β-actin signal was accumulated in a complex pattern in the apical region (Figure 4.1). Both anti-aPKC-ı and anti-ezrin signals, accumulated regionally at the apical membrane in 7-8 week ST with a significantly increased colocalization compared to 4-6 weeks gestation (Figure 4.1 A-B; E), although there were areas consistently observed where an apical anti-ezrin signal was absent in all samples examined. Quantification of aPKC-ı: ezrin colocalization coefficients for 9-12 weeks and 37–40-weeks (term) ST, revealed that colocalization signal was more consistently apical between biological replicates and was significantly increased compared to 4-6 weeks (Figure 4.1C-D; E). Similarly, compared to 4-6 weeks ST a significant increase in the apical aPKC-ζ: ezrin colocalization coefficient by 7-8 weeks gestation was observed (Figure 4.2 A-B; E). Highly consistent colocalization of anti-aPKC-ζ and anti-ezrin signal was also observed at 9-12 weeks and 37-40 weeks gestation (Figure 4.2 C-D; E). These data reveal a consistent high degree of spatial proximity of aPKC isoforms and ezrin in the apical region of the ST by 9-12 weeks gestation.

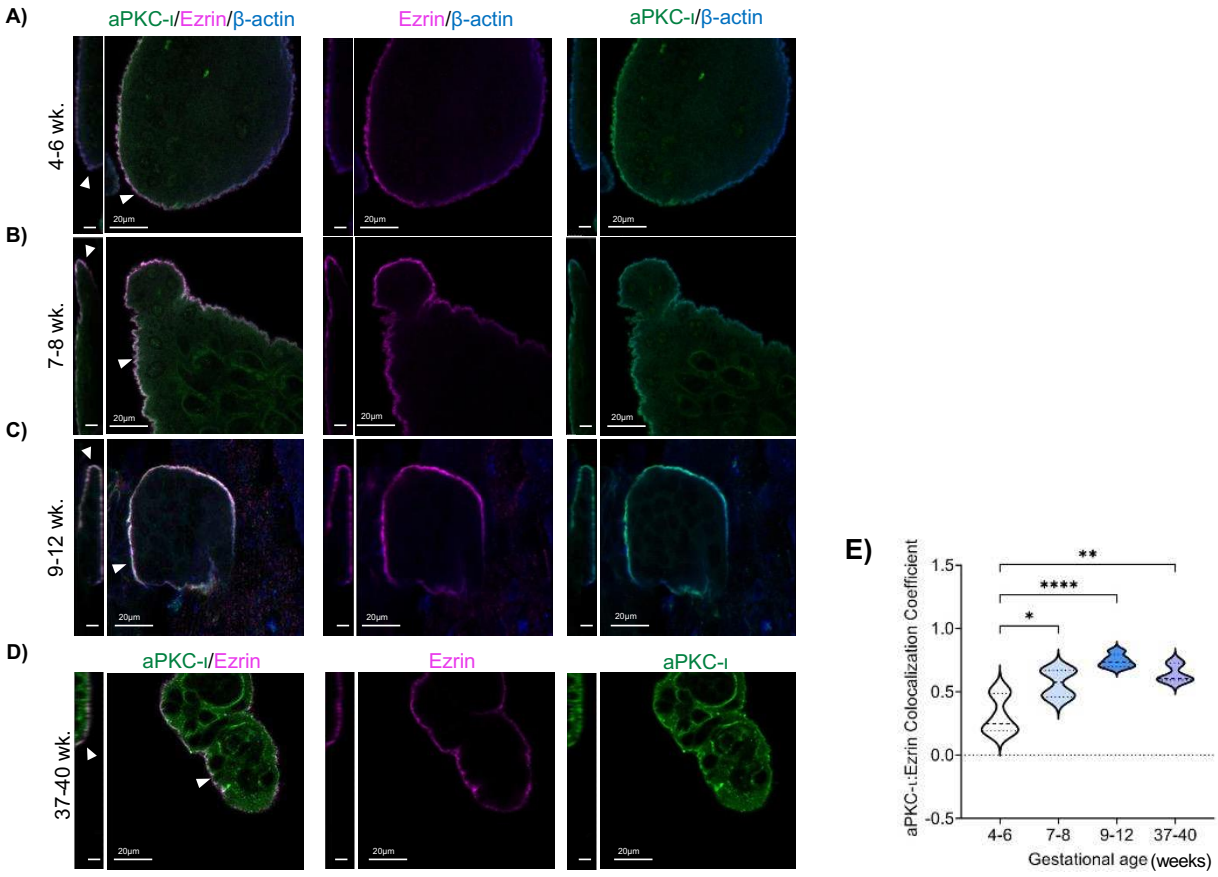


Figure 4.1: aPKC-ι colocalizes strongly with ezrin at the ST apical surface in 9-12 weeks and 37-40 weeks ST.

Representative images of A) 4-6 week B) 7-8 week C) 9-12 week stained with anti-aPKC-ι (green), anti-ezrin (magenta) and anti β-actin (blue); left panel=zy plane scale bar=10μM; right panel=xy plane; arrowheads=ST apical surface; single image plane of z-stack images and D) 37-40 week placental tissue stained with anti-aPKC-ι (green), anti-ezrin (magenta); left panel=zy plane scale bar=10μM; right panel=xy plane; arrowheads=ST apical surface; single image plane of z-stack images; E) Summary data for quantitation of Pearson's colocalization coefficient for aPKC-ι and ezrin at the ST apical surface through varying gestational ages; *n*=3; *=p<0.05; **=p<0.01, ***=p<0.001; bold dashed line=median; dotted line = interquartile range. Analyses performed with one-way ANOVA with Tukey's post-hoc test. (22)[9]

[9] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.1A-B; Fig.S1). In *pre-print. Biorxiv*. 2022

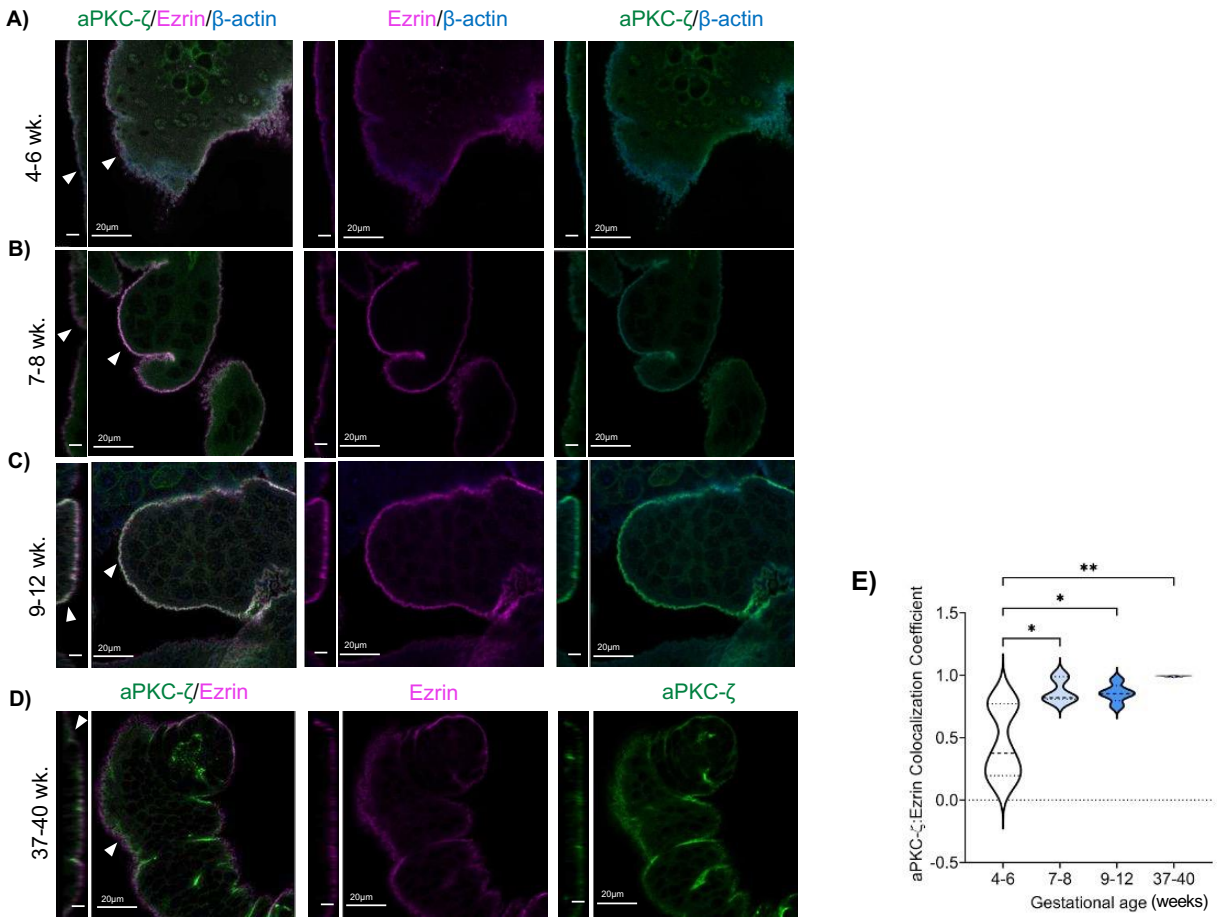


Figure 4.2: aPKC-ζ strongly colocalizes with ezrin at the ST apical surface in 9-12 weeks and 37-40 weeks ST.

Representative images of A) 4-6 week B) 7-8 week C) 9-12 week stained with anti-aPKC-ζ (green), anti-ezrin (magenta) and anti β-actin (blue); left panel=zy plane, scale bar=10μM; right panel=xy plane; arrowheads=ST apical surface; single image plane of z-stack images and D) 37-40 placental explants stained with anti-aPKC-ζ (green), anti-ezrin (magenta) left panel=zy plane scale bar=10μM; right panel=xy plane; arrowheads=ST apical surface; single image plane of z-stack images; E) Summary data for quantitation of Pearson's colocalization coefficient for aPKC-ζ and ezrin at the ST apical surface through varying gestational ages; *n*=3; *=p<0.05; **=p<0.01; bold dashed line=median; dotted line = interquartile range. Analyses performed with one-way ANOVA with Tukey's post-hoc test. (22)[10]

[10] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.1C-D; Fig.S1). In *pre-print. Biorxiv*. 2022

As mentioned above, ezrin needs to be activated via phosphorylation for it to link the F-actin and microvillar membrane. Previous studies revealed aPKCι/λ mediated phosphorylation of ezrin at Thr567 residue during murine intestinal development. Thus, we wanted to investigate if similarly, aPKCs are responsible for activating ezrin at the ST apical membrane. For this, we used late first-trimester placental explants (9-12 weeks) and term explants (37-40 weeks) and blocked the kinase activity of both aPKC-ι and aPKC-ζ isoforms using a myristoylated aPKC inhibitor (For methods see section 2.3.1) and analyzed the tissue using anti-phospho Thr567 ezrin and total ezrin (For methods see section 2.5). With inhibitor treatment, a significant decrease in the anti-phospho-Thr-567 ezrin signal relative to the total ezrin signal was observed at the ST apical membrane (Fig 4.3A-B; 4.4A-B). We also observed that the total anti-ezrin signal significantly decreased at the ST apical membrane in both 9-12 week and 37-40 week aPKC inhibitor treated explants compared to control explants after 6hrs of aPKC inhibitor treatment (Fig 4.3C; Fig 4.4C). This suggests that reduced aPKC kinase activity not only decreases ezrin phosphorylation apically but can also mislocalize ezrin from the ST apical membrane. Thus, these data show that aPKC kinase activity regulates the apical expression of ezrin as well as its phosphorylation at Thr-567 in the human ST.

A visible change in the ST apical β-actin staining pattern was observed with aPKC inhibitor treatment (Fig 4.3- 4.4). Thus, we examined if loss of aPKC kinase activity alters the ST apical F-actin using phalloidin. We quantified apical phalloidin signal intensity after 2, 4 and 6hrs of aPKC inhibitor treatment in the late first trimester (9-12 weeks) (For methods see section 2.3.1; 2.5) (Fig 4.5 A-C; E-G). Inhibitor treatment led to regions with a significant decrease in the phalloidin signal

intensity at the ST apical surface in the late first trimester (9-12 weeks) placental explants after 2 and 4hrs treatment (Figure 4.5 A-B; E-F). With a consistent decrease

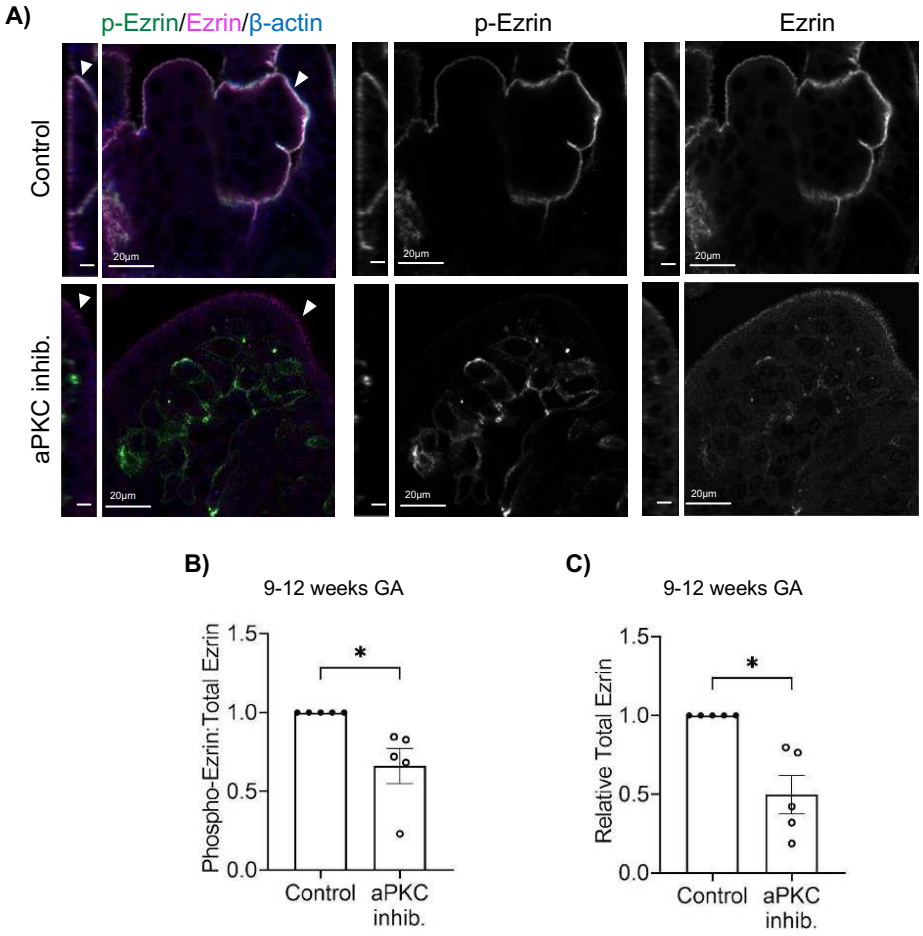


Figure 4.3: aPKC activity regulates ST apical ezrin activation and abundance in 9-12 weeks placental explants.

A) Representative images of 9-12 week placental explants treated +/- aPKC inhibitor for 6 hrs stained for anti-phospho(Thr567)-ezrin (green), anti-ezrin (magenta) and anti-β-actin (blue); left panel=zy plane scale bar=20μM; right panel=xy plane; arrowheads=ST apical surface; single image plane of z-stack images; B) Summary data for quantitation of ST apical phospho(Thr567) ezrin relative to total ezrin signal intensity in 9-12 week explants; C) Summary data for

quantitation of ST apical ezrin signal intensity in 9-12 week explants; *n*=5; Analyses performed using one-sample t-test; *=p<0.05 mean+/- S.E.M. (22)[11]

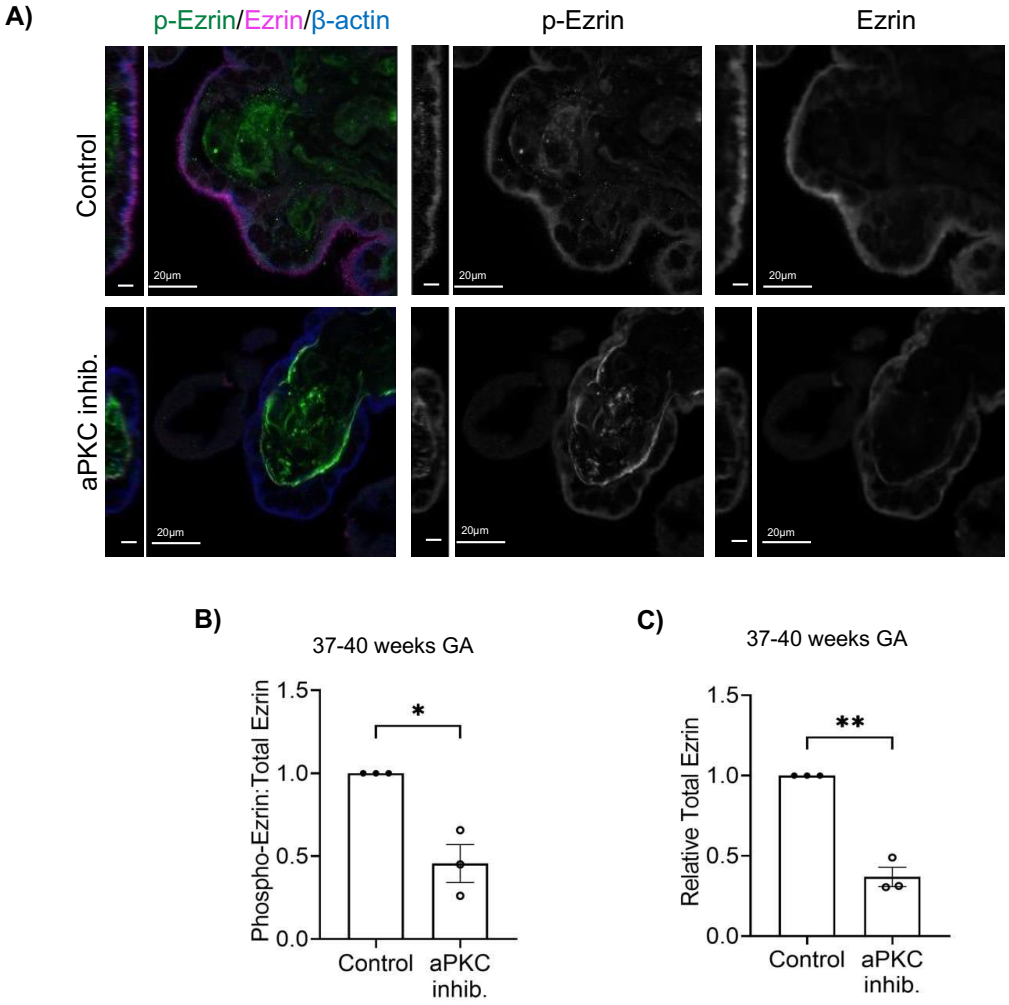


Figure 4.4: aPKC activity regulates ST apical ezrin activation and abundance in 37-40 weeks placental explants.

A) Representative images of 37-40 week placental explants treated +/- aPKC inhibitor for 6 hrs stained for anti-phospho(Thr567)-ezrin (green), anti-ezrin (magenta) and anti-β-actin (blue); left panel=zy plane scale bar=20μM; right panel=xy plane; single image plane of z-stack images; B) Summary data for quantitation of ST apical phospho(Thr567)

[11] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.2A). In *pre-print. Biorxiv*. 2022

ezrin relative to total ezrin signal intensity in 37-40 week explants; C) Summary data for quantitation of ST apical ezrin signal intensity in 37-40 week explants; *n*=3; Analyses performed using one-sample t-test; *=p<0.05 mean+/- S.E.M. (22)[12]

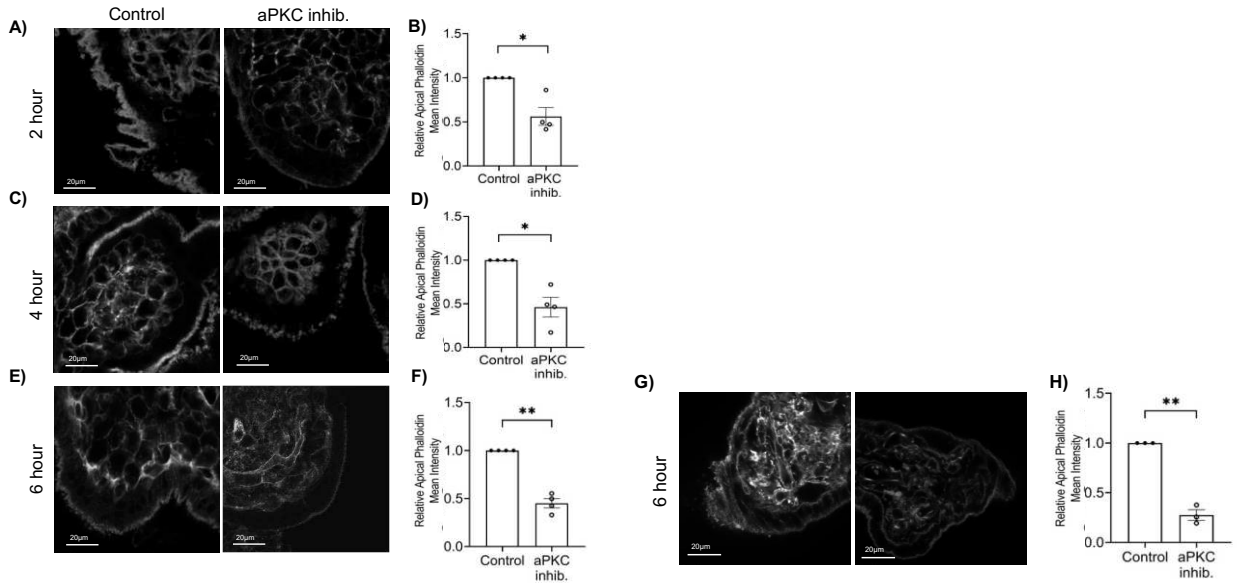


Figure 4.5: aPKC inhibitor treatment alters apical F-actin abundance in both first trimester (9-12 week) and term (37-40 weeks) placental explants.

A) Representative images of 9-12 week placental explants treated +/- aPKC inhibitor for 2 hrs stained with phalloidin (grayscale) ; scale bar=20μM; xy plane; single image plane of z-stack images and B) Summary data for quantitation of ST apical phalloidin signal intensity in 9-12 week explants for 2 hrs; n=4; *=p<0.05 C) Representative images of 9-12 week placental explants treated +/- aPKC inhibitor for 4 hrs stained with phalloidin (grayscale) ; scale bar=20μM; xy plane; single image plane of z-stack images and D) Summary data for quantitation of ST apical phalloidin signal intensity in 9-12 week explants for 4 hrs; n=4; *=p<0.05 E) Representative images of 9-12 week placental explants treated +/- aPKC inhibitor for 6 hrs stained with phalloidin (grayscale) ; scale bar=20μM; xy plane; single image plane of z-stack images and F) Summary data for quantitation of ST apical phalloidin signal intensity in 9-12 week explants for 6 hrs; n=4 **=p<0.01; G) Representative images of 37-40 week placental explants treated +/- aPKC inhibitor for 6 hrs stained with phalloidin (grayscale) ; scale bar=20μM; xy plane; single image plane of z-stack images and H)

[12] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig. S2). In *pre-print. Biorxiv*. 2022

Summary data for quantitation of ST apical phalloidin signal intensity in 37-40 week explants for 6 hrs; n=3 ;*=p<0.05,**=p<0.01. All above analyses performed using one-sample t-test; summary graphs mean +/- S.E.M. (22)[13]

of approximately 50% after 6 hrs of aPKC inhibitor treatment in both late first trimester (9-12 weeks) and term (37-40 weeks) placental explants (Fig 4.5 C-D; G-H). Interestingly, regionalized effect of aPKC inhibition on F-actin alterations was noted, with regions of appreciably decreased phalloidin signal adjacent to areas with negligible change in apical phalloidin intensity. Thus, these data suggest that aPKC kinase activity regulates ST F-actin abundance at the end of first trimester and term.

To examine if there are isoform-specific functions of aPKCs and to address potential off-target effects of aPKC inhibitor, we performed *PRKC* isoform targeted siRNA knockdown (For methods see section 2.3.2). First trimester (9-12 weeks) placental tissue was treated for single isoform knockdown with siRNA targeting either *PRKCI* or *PRKCZ* for 24 hours. Additionally, we also performed double isoform knockdown with siRNAs targeting both *PRKCI* and *PRKCZ* for 24 hours. For this, we performed western blot analyses of explant lysates to determine the knockdown efficiency for aPKC-ι, aPKC-ζ, and aPKC-ζ III (For methods see section 2.7) (Figure 4.6A; 4.7A; 4.8A). We achieved consistent knockdown efficiency of approximately 35% when explants were treated with siRNA. Treatment with siRNA targeting *PRKCZ* led to the knockdown of both the ζ isoforms: aPKC-ζ, and aPKC-ζ III (Fig 4.7A; C, Fig 4.8A; C). It is also important to note that our siRNA knockdown method targets siRNA efficiently to the ST, however it may lead to a limited, but variable, siRNA accumulation in the underlying cytotrophoblast progenitor cells

[13] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.2F; Fig. S4). In *pre-print. Biorxiv*. 2022

which are also known to express all three aPKC isoforms (23). We also performed knockdown using a second set of siRNAs targeting *PRKCI* or *PRKCZ* (Fig 4.6C-D; Fig 4.7D-F). Double isoform knockdown was carried out only siRNA- PRKCI#1 and siRNA- PRKCZ#1 because the mean knockdown efficiency siRNA- PRKCI#1 was 36% vs. 21% for siRNA- PRKCI#2 (Fig. 4.6). Similarly, mean knockdown efficiency of siRNA- PRKCZ #1 was 32% with a relatively consistent knockdown for the full length aPKC-ζ vs. 28% for siRNA- PRKCZ#2 (Fig. 4.7A-B; D-E). Additionally, the siRNA knockdown of the aPKC-ζ III isoform was consistent only with siRNA - *PRKCZ#2* (Fig. 4.7C and 4.7F).

After confirming sufficient knockdown efficiency, we went on to investigate if the knockdown of a single or both aPKC isoforms alter the ST apical F-actin in siRNA-treated explants using phalloidin (For methods see section 2.3.2; 2.7; 2.5). Additionally, we used wheat germ agglutinin (WGA-lectin; Figure 4.9;4.10), a lectin binding to the ST apical surface and cytoplasm to better visualize the ST, when ST F-actin decreases (24). We saw a significantly decreased ST apical phalloidin (~70%) when explants were treated with *PRKCI* siRNA, *PRKCZ* siRNA, or both (Figure 4.9) thereby suggesting that the ST apical membrane F-actin abundance is regulated by both the aPKC-ı and aPKC-ζ isoforms and decreased expression of even a single isoform is sufficient to alter the ST apical actin cytoskeletal dynamics. Importantly, similar to what we observed with aPKC inhibitor treatment, we saw a regionally specific appearance of phalloidin replete areas with single isoform knockdown as well as a double knockdown. Supporting this data, we also observed a similar effect on F-actin when the second set of siRNA was used for single isoform knockdown (Figure 4.10).

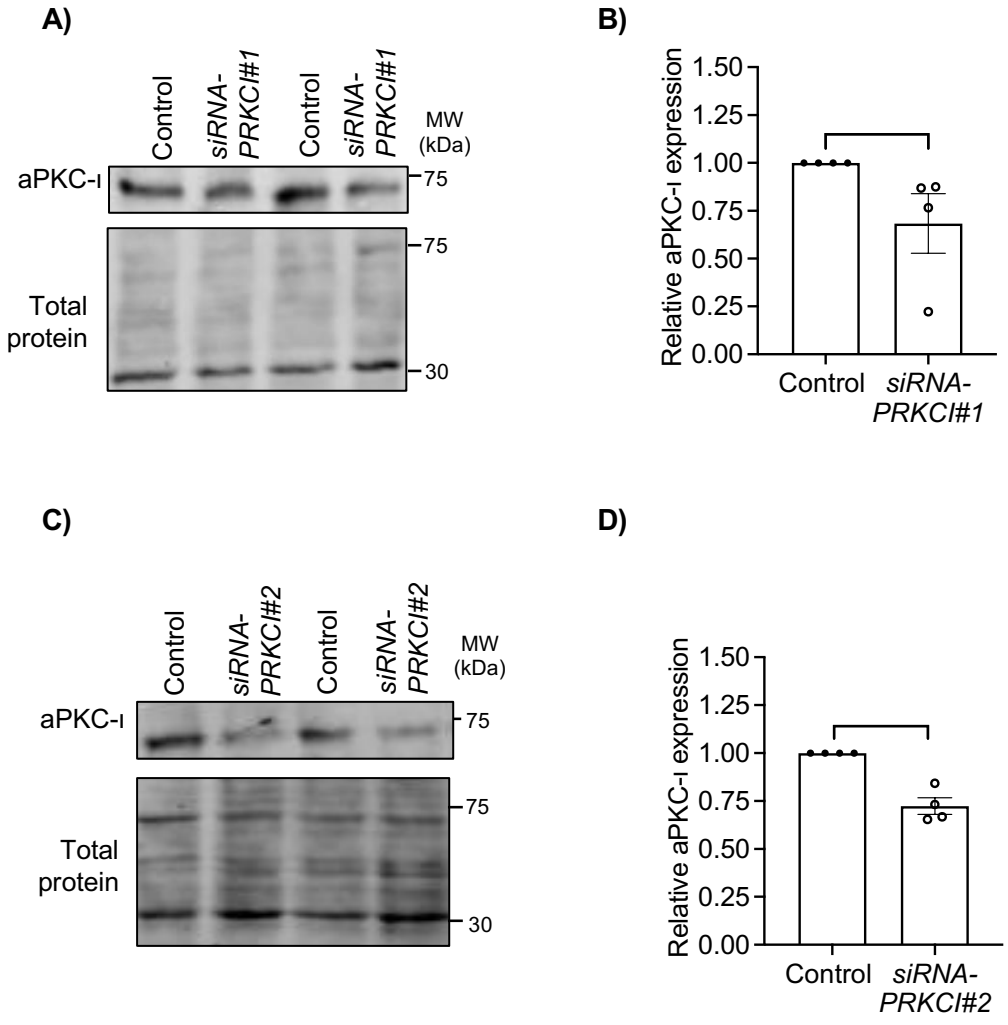


Figure 4.6: PRKCI targeting siRNA knockdown efficiency in first trimester explants (9-12 weeks)

A) Representative western blot with anti-aPKC-ι and total protein following siRNA#1 knockdown targeting *PRKCI* in 9-12 week explant lysates. B) Summary data for quantitation of relative aPKC-ι expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#1 C) Representative western blot with anti-aPKC-ι and total protein following siRNA#2 knockdown targeting *PRKCI* in 9-12 week explant lysates. D) Summary data for quantitation of relative aPKC-ι expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#2. All above analyses performed using one-sample t-test; summary graphs mean +/- S.E.M (22)[14]

[14] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.2I; 2L). In *pre-print. Biorxiv*. 2022

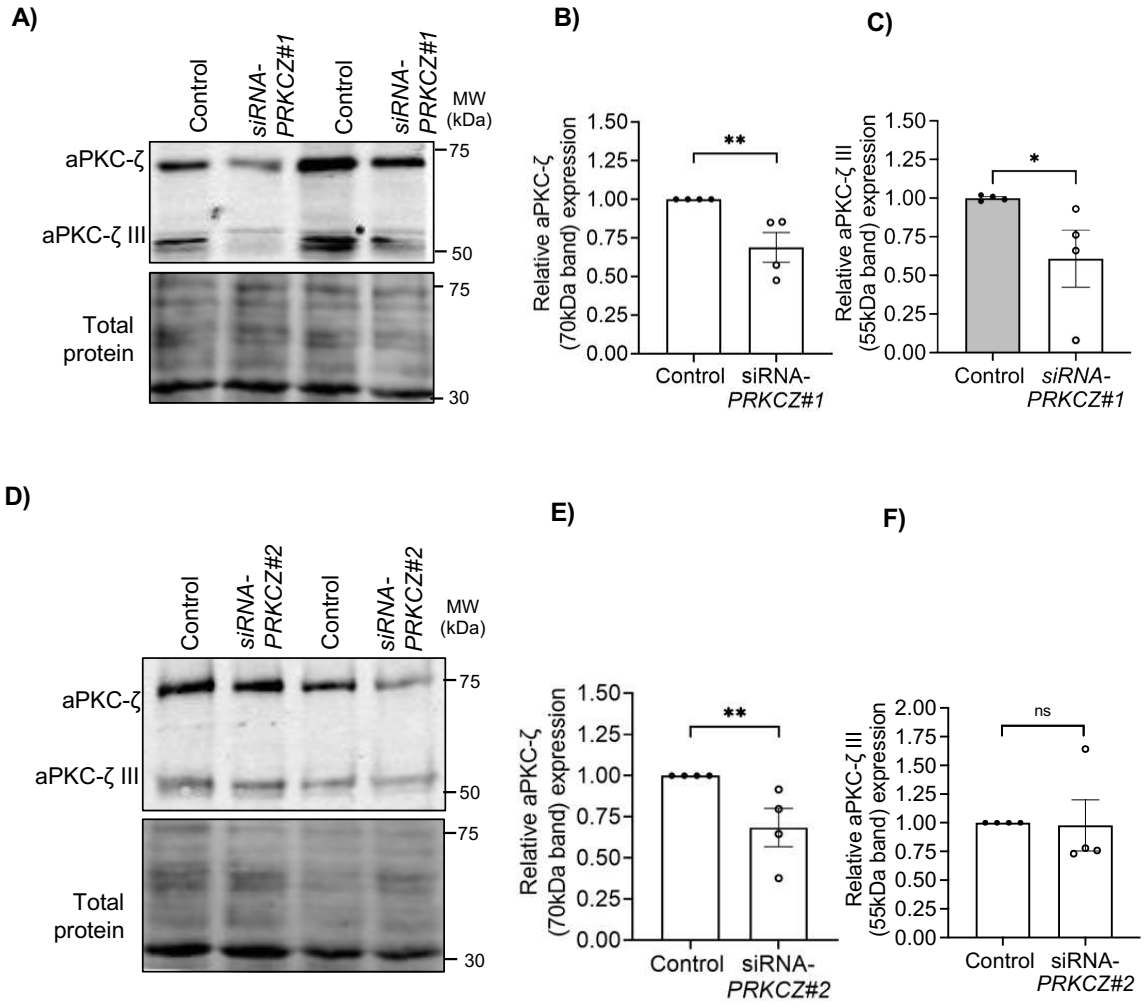


Figure 4.7: PRKCZ targeting siRNA knockdown efficiency in first trimester explants (9-12 weeks)

A) Representative western blot with anti-aPKC-ζ and total protein following siRNA#1 knockdown targeting *PRKCZ* in 9-12 week explant lysates. B) Summary data for quantitation of relative aPKC-ζ expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#1 C) Summary data for quantitation of relative aPKC-ζIII expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#1. D) Representative western blot with anti-aPKC-ζ and total protein following siRNA#2 knockdown targeting *PRKCZ* in 9-12 week explant lysates. E) Summary data for quantitation of relative aPKC-ζ expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#2 F) Summary data for

quantitation of relative aPKC-ζIII expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#2. All above analyses performed using one-sample t-test; summary graphs mean +/- S.E.M(22)[15]

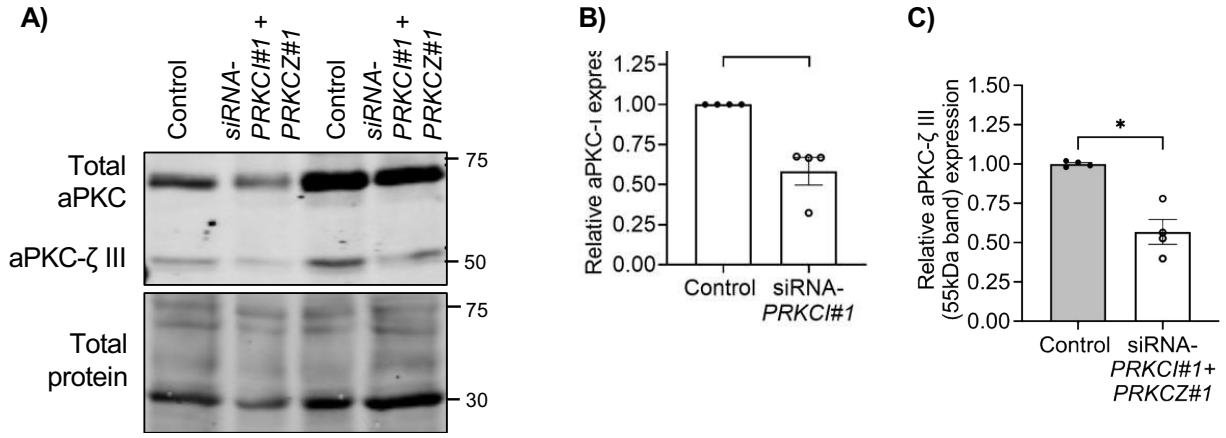


Figure 4.8: PRKCI+PRKCZ targeting siRNA knockdown efficiency in first trimester explants (9-12 weeks).

A) Representative western blot with anti-total-aPKC and total protein following siRNA#1 knockdown targeting *PRKCI* and *PRKCZ*; in the 9-12 week explant lysates B) Summary data for quantitation of relative aPKC-ζ expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#1 targeting both *PRKCI* and *PRKCZ* C) Summary data for quantitation of relative aPKC-ζIII expression in 9-12 week explant lysates after 24 hrs treatment with siRNA#1 targeting both *PRKCI* and *PRKCZ*. All above analyses performed using one-sample t-test; summary graphs mean +/- S.E.M(22)[16]

[15] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.2J; 2M-N). In *pre-print. Biorxiv*. 2022

[16] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.2K; 2O-P). In *pre-print. Biorxiv*. 2022

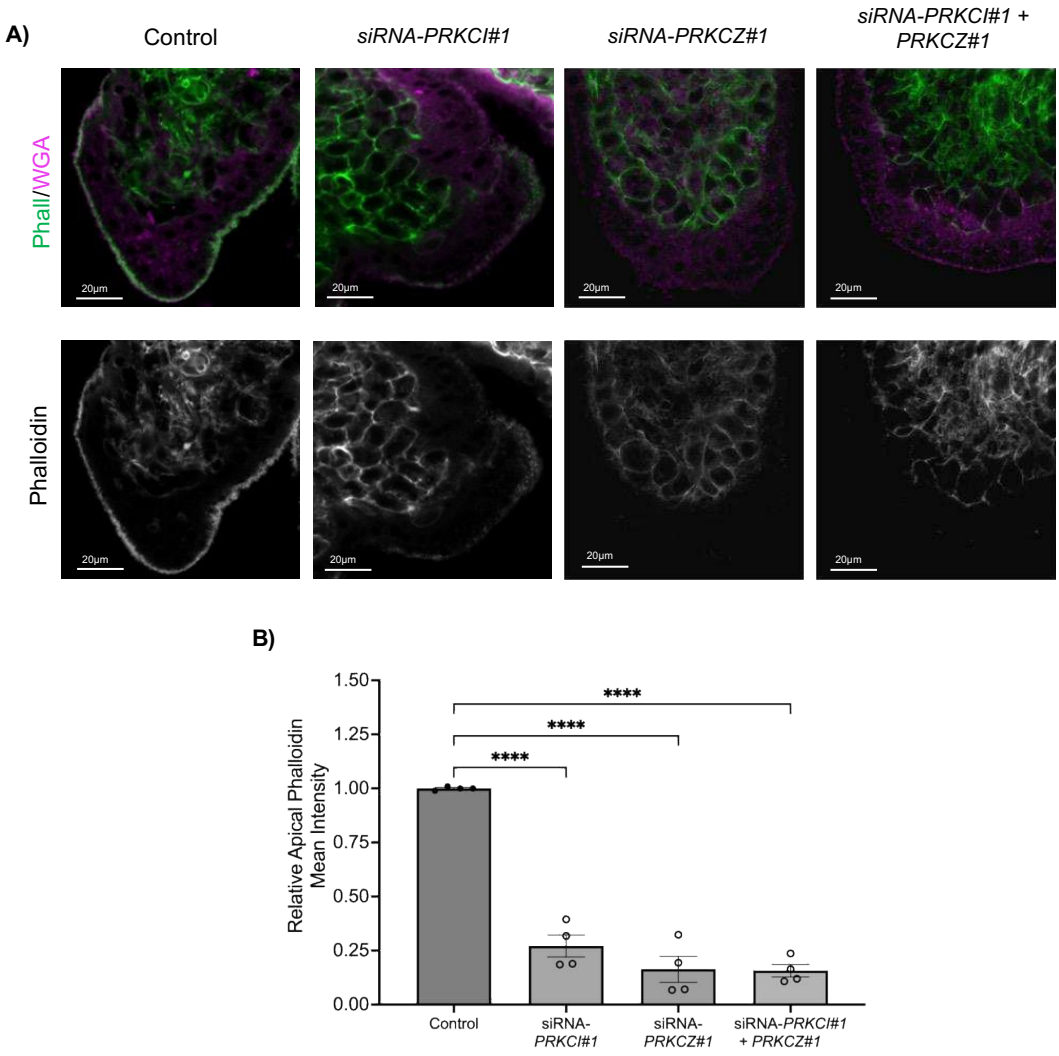


Figure 4.9: siRNA knockdown targeting either PRKCI or PRKCZ isoforms alter apical F-actin abundance in 9-12 week placental explants.

Representative images of 9-12 week placental explants treated with siRNA#1 targeted for Scramble (control) *PRKCI; PRKCZ; PRKCI +PRKCZ* for 24 hrs stained with phalloidin (green) and WGA-lectin (magenta); scale bar=20µM; xy plane; single image plane of z-stack images; B) Summary data for quantitation of ST apical phalloidin signal intensity in 9-12 week explants; *n*=4;****=p<0.0001; Analyses performed using one-way ANOVA with Dunnett's post-hoc test; summary graphs mean +/- S.E.M (22)[17]

[17] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.2Q). In *pre-print. Biorxiv*. 2022

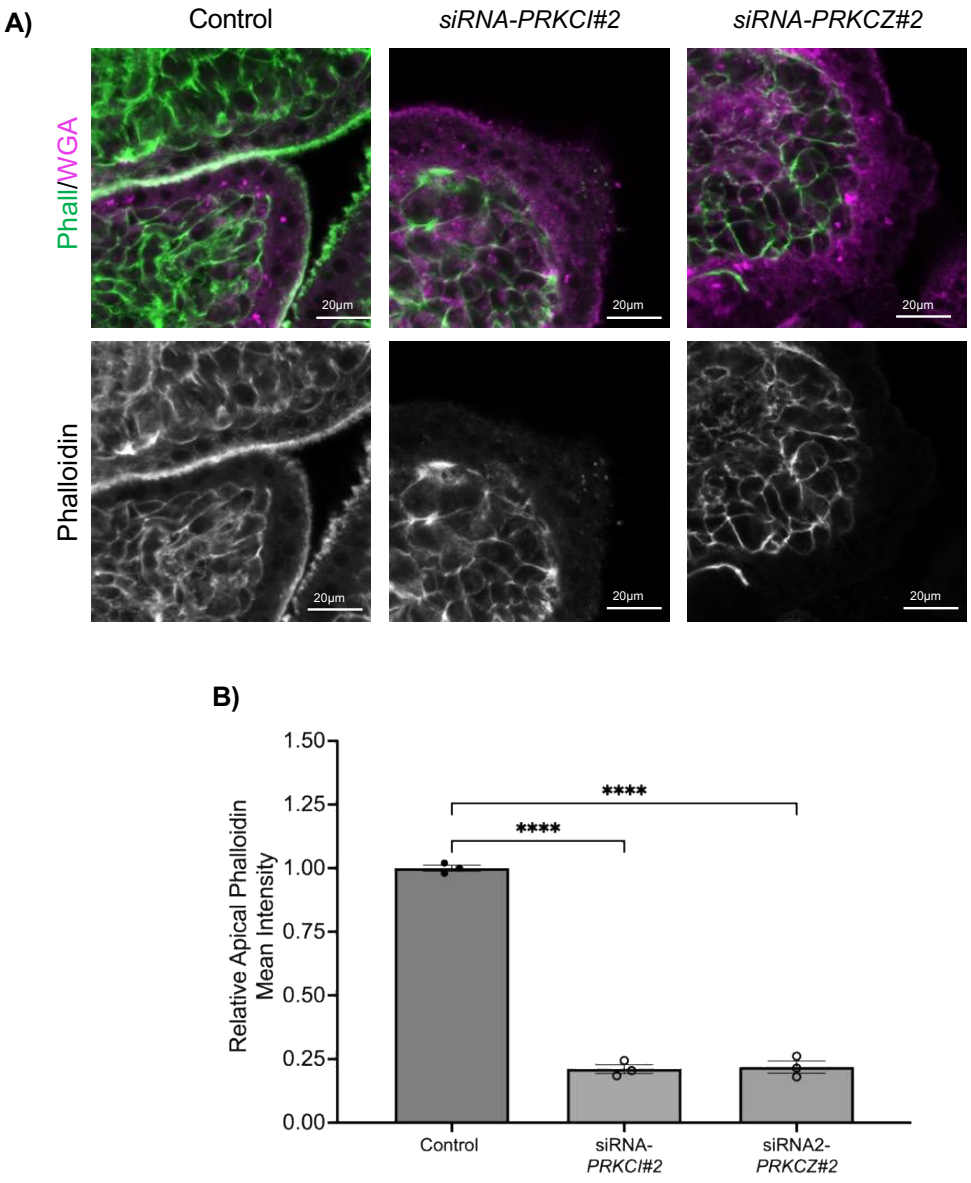


Figure 4.10: Second set of siRNA targeting either PRKCI or PRKCZ isoforms decreases apical F-actin abundance in 9-12 week placental explants.

Representative images of 9-12 week placental explants treated with siRNA#2 targeted for Scramble (control) *PRKCI; PRKCZ;* for 24 hrs stained with phalloidin (green) and WGA-lectin (magenta); scale bar=20µM; xy plane; single image plane of z-stack images; B) Summary data for quantitation of ST apical phalloidin signal intensity in 9-12 week explants; *n*=4;****=p<0.0001; Analyses performed using one-way ANOVA with Dunnett's post-hoc test; summary graphs mean +/- S.E.M (22)

To confirm if loss of apical F-actin alters the ST apical microvilli and apical surface structure we performed electron microscopy on first trimester explants (9-12 weeks) treated +/- aPKC inhibitor (For methods see section 2.6). SEM of aPKC inhibitor treated explants revealed a severe loss of microvilli abundance with sparse microvilli at the ST apical surface and these remaining microvilli coalesced with an overall porous appearance of the apical membrane (Figure 4.11) in a region-specific manner. TEM further confirmed the loss of ST microvilli observed by SEM. Additionally, TEM revealed an ST-specific loss of cytoplasmic density and an increase in the number of apically localized membrane-coated vacuoles of variable sizes in addition to the presence of swollen mitochondria (Figure 4.12). Critically, while performing TEM we did not observe visible changes in the underlying cytotrophoblast progenitor cells.

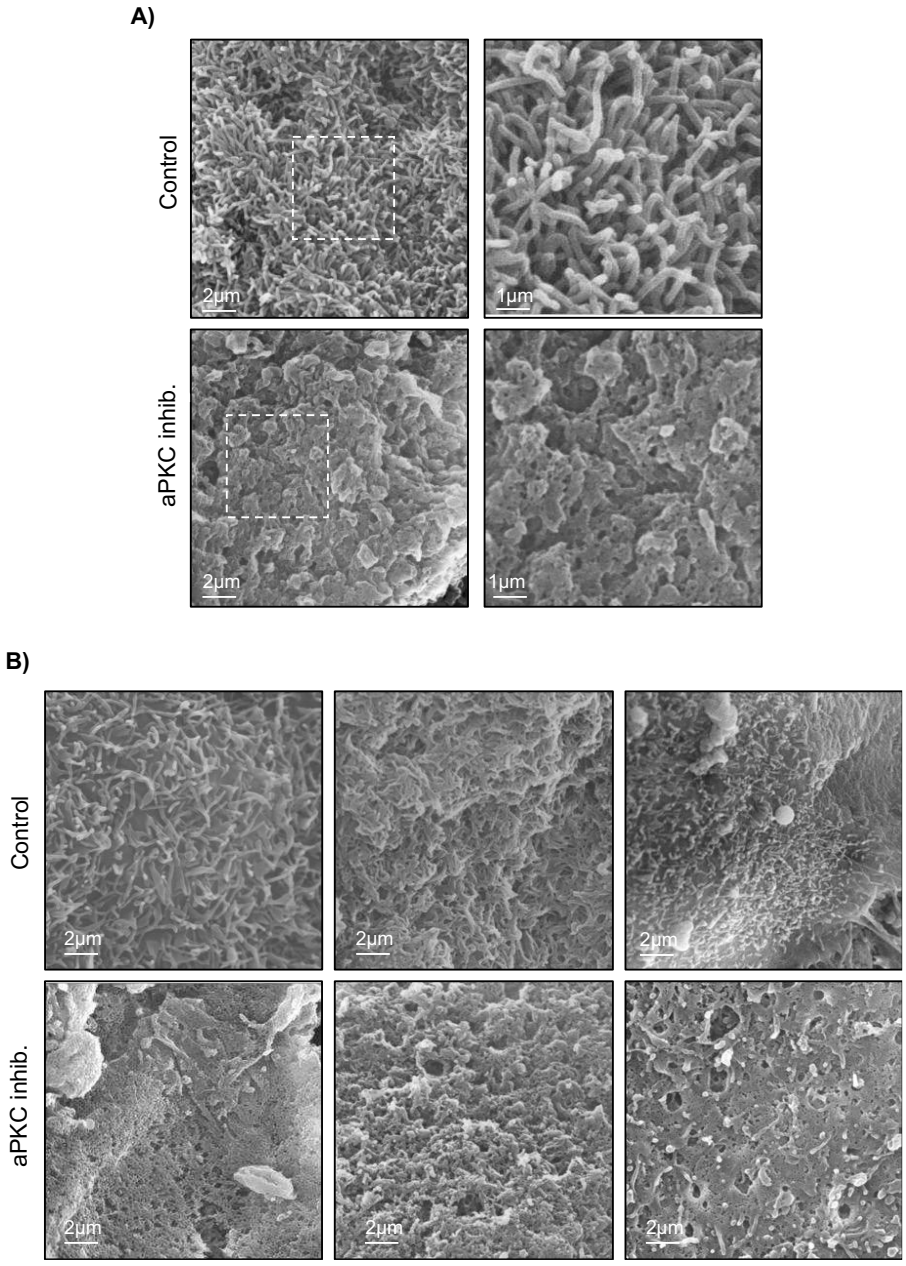


Figure 4.11: SEM images of 9-12 week placental explants treated with aPKC inhibitor reveal decreased apical microvilli.

A) Representative SEM images of 9-12 week placental explants treated with aPKC inhibitor for 6hrs (representative of *n*=3); right panels=isolated zoomed images of boxed area indicated on left. B) Additional representative SEM images of 9-12 week placental explants treated with aPKC inhibitor for 6hrs. (22)[18]

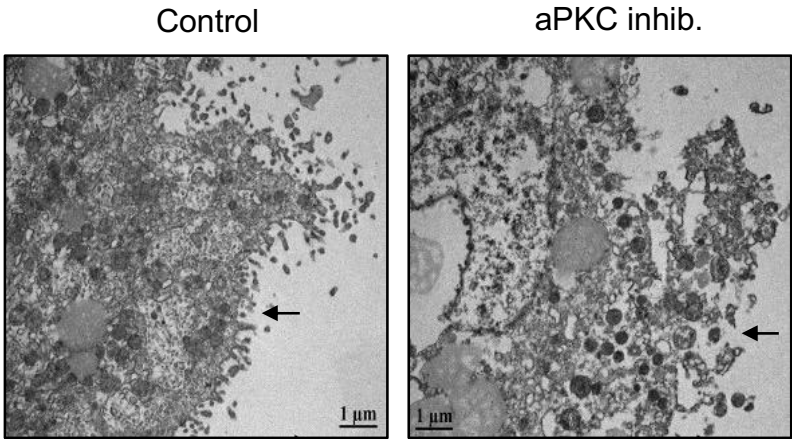


Figure 4.12: aPKC inhibition leads to simplification of ST apical membrane, decreased cytoplasmic density

A) Representative TEM images of 9-12 week placental explants treated +/- aPKC inhibitor for 6hrs (representative of *n*=3). Arrows represent ST apical surface (22)[19]

EM imaging revealed an almost porous appearance of the apical membrane and a regionalized decrease in the ST cytoplasmic density hence we reasoned that there is a possibility that aPKC kinase inhibition increases ST permeability. To test ST membrane permeability, we inhibited total aPKC kinase activity as well as performed siRNA knockdown in the first trimester (9-12 weeks) of human placental explants and examined the uptake of fluorescently conjugated dextran, a membrane-impermeable glycan that can be used to assess membrane integrity. For this, we pulsed explants with 10,000 Da neutral dextran following 6 hrs of aPKC inhibitor treatment and

[18] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.3A and Fig.S5). In *pre-print. Biorxiv*. 2022

[19] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.3B). In *pre-print. Biorxiv*. 2022

24 hrs of siRNA knockdown (For methods see section 2.3; 2.4). Our data reveal that the decrease in aPKC kinase activity or expression by siRNA knockdown leads to a 4-fold and 2.5-fold increase respectively in the uptake of neutral dextran (Figure 4.13A-B, 4.14A-B). Moreover, hyperpermeability of the ST to dextran, was restricted to regions of the ST with appreciably decreased apical F-actin (Figure 4.13A, 4.14A). Notably, in control explants, a punctate dextran signal localized to the apical surface of the ST consistent with macropinocytotic uptake was observed, whereas the dextran signal for aPKC inhibitor and siRNA treated explant was relatively diffuse throughout the cytoplasm. Thus, taken together, the SEM images and these data show that decreased aPKC isoform kinase activity or expression disrupts ST apical membrane integrity and permeabilizes the ST to a neutrally charged 10,000-dalton compound.

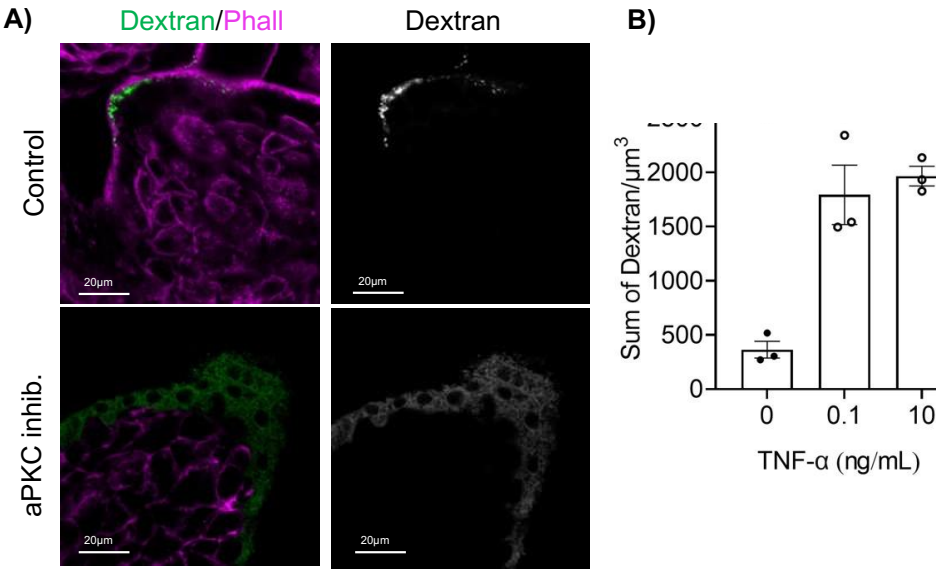


Figure 4.13: aPKC inhibition permeabilizes ST to 10000 MW neutral compound.

A) Representative images of 9-12 week placental explants after 10,000 MW dextran-Texas Red uptake (green) and phalloidin staining (magenta) following 2hrs treatment with aPKC inhibitor; left panels=merged image; right panels=isolated dextran signal; single plane of z-stack images; B) Summary data for quantitation of sum of 10,000 MW

dextran-Texas Red per μm^3 in 9-12 week placental explants after 2hrs aPKC inhibitor treatment; *n*=4; *=p<0.05; Student's t-test. (22)[20]

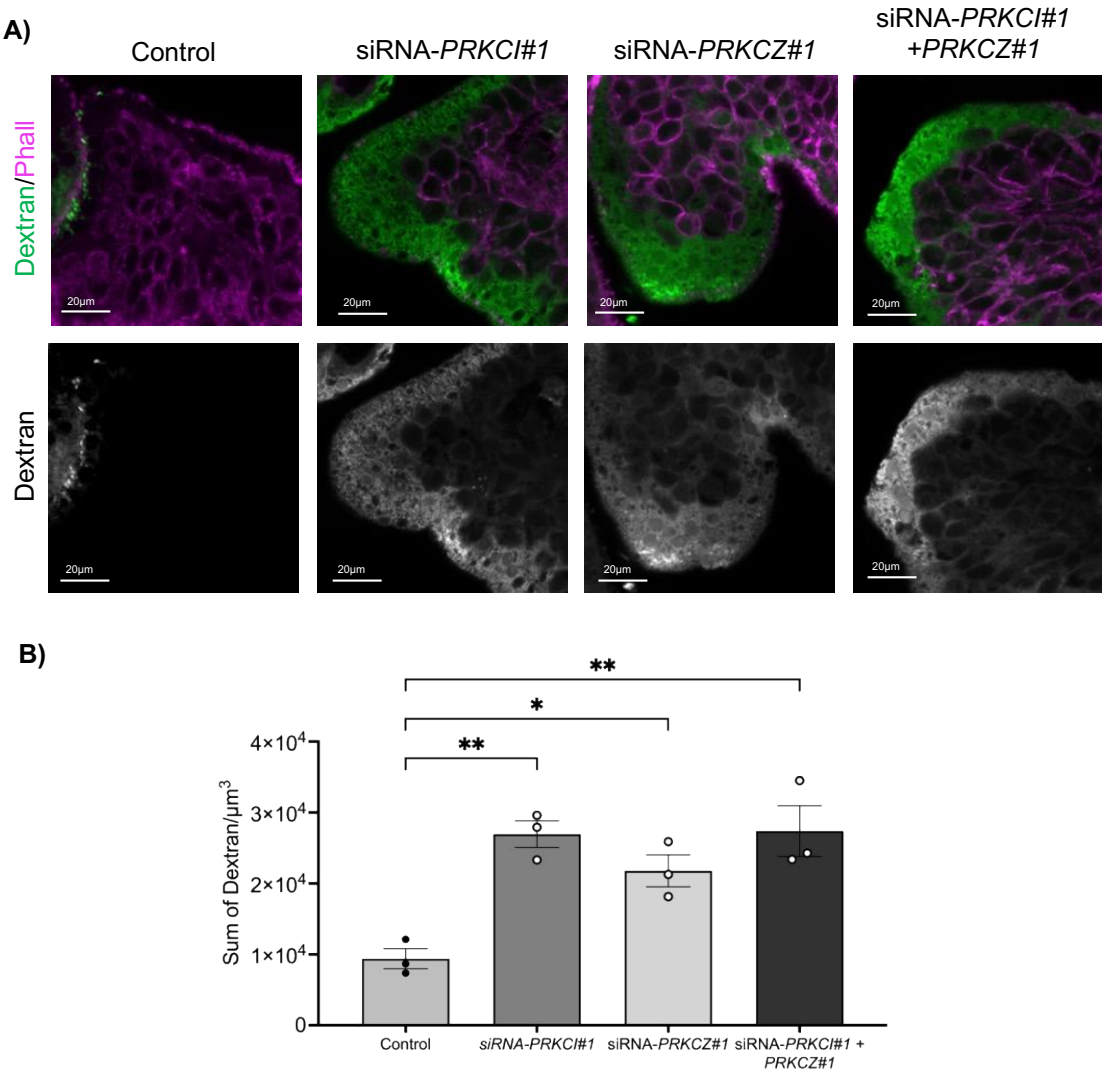


Figure 4.14: siRNA knockdown targeting either PRKCI or PRKCZ isoforms permeabilizes the ST to 10000 MW neutral compound.

A) Representative images of 9-12 week placental explants after 10,000 MW dextran-Texas Red uptake (green) and phalloidin staining (magenta) following 24hrs treatment with scramble control or siRNA sequences targeting *PRKCI* and/or *PRKCZ*; top panels=merged image; bottom panels=isolated dextran signal; single image plane of z-stack

[20] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.3C-D). In *pre-print. Biorxiv*. 2022

images; B) Summary data for quantitation of sum of 10,000 MW dextran-Texas Red per μm^3 in 9-12 week placental explants following siRNA knockdown of *PRKCI* and/or *PRKCZ*; *n*=3; *=p<0.05, **=p<0.01; one-way ANOVA with Dunnett's post-hoc test(22)[21]

Since aPKC inhibitor treatment increased membrane permeability, we hypothesized that this may also cause an increased release of ST cytoplasmic contents. The ST is known to produce various pregnancy-supporting hormones, including the hormone human chorionic gonadotropin (hCG). Therefore, we quantified the release of the hCG-β subunit in the explant conditioned medium of control vs. aPKC inhibitor-treated samples via ELISA (For methods see section 2.9). As expected, there was a nearly 3-fold increase in the extracellular release of hCG-β in explant conditioned medium of first trimester explants treated with aPKC inhibitor for 6hrs (Figure 4.15).
Combined together, the dextran uptake and the hCG release suggest that there is altered ST permeability and the cytoplasmic contents are released when aPKC kinase activity is inhibited.

[21] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.3E-F). In *pre-print. Biorxiv*. 2022

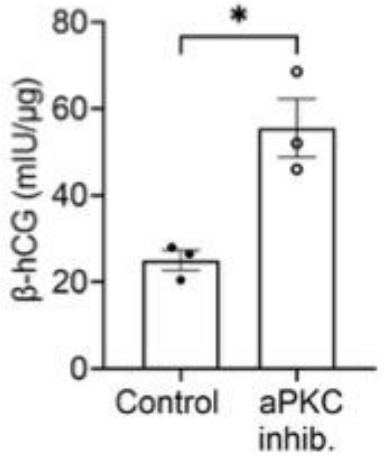


Figure 4.15: Extracellular release of human chorionic gonadotropin β sub-unit increases after 6 hours of aPKC inhibitor treatment.

Summary data for human chorionic gonadotropin β sub-unit release measured in mIU/µg via ELISA in 9-12 week placental explants after 6 hrs aPKC inhibitor treatment, n=3, *=$p<0.05$; Student's t-test.(22)[22]

4.4 Discussion

The fetally derived human ST and its microvilli are bathed in the maternal blood making it the prime interface for the maternal-fetal exchange. We found that aPKC isoforms are critical regulators of ST structure, permeability, and function in a spatially restricted manner. While we established that aPKCs accumulate apically in the human ST, the mechanism by which it maintains the ST polarity is not yet known. The scaffolding protein Par-6 responsible for localizing aPKC kinase apically has been shown to be expressed at the apical surface around 12 weeks of gestation, which coincides with the strong apical accumulation of aPKC isoforms in the ST (Figure 4.1 and Figure 4.2) (25). However, the expression and localization of Par 3 within the ST are not yet known. Thus, it is unclear whether aPKC isoforms function as a part of the Par complex in the

[22] Modified from Patel *et.al.* Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface (Fig.3I). In *pre-print. Biorxiv*. 2022

ST. Critically, the apical localization of aPKC-ι regulated ezrin phosphorylation in intestinal microvilli(13). Our data revealed a strong colocalization between aPKC isoforms and ezrin at the ST apical membrane. The established spatial proximity suggests that aPKCs may directly phosphorylate ezrin at the ST apical membrane to maintain microvillar integrity. Additionally, to our knowledge, our data is the first to show the apical expression of ezrin in the first-trimester placental syncytiotrophoblast.

Our experiments indicate that aPKC kinase activity contributes to the phosphorylation of apical ezrin at Thr567 and that blocking aPKC kinase activity not only significantly decreased ezrin phosphorylation but also led to an overall decrease of total ezrin expressed at the ST apical membrane. Studies have shown that ezrin undergoes a cycle of proteasomal degradation and resynthesis based on cellular demand (26). This loss of total ezrin in the absence of aPKC kinase activity could either be due to mislocalized ezrin or it could also be due to less apical recovery of dephosphorylated ezrin. However, one of the limitations of our study is that aPKC isoforms are known to integrate with various other polarity proteins and complexes to carry out their functions (27–29). Our study doesn't account for any other proteins that might directly or indirectly associate with aPKCs to phosphorylate ezrin apically. Thus, additional research is required to elucidate the mechanism by which aPKCs regulate ezrin in the ST. Importantly, our data suggests that the phosphorylation and apical localization of ezrin is only partially dependent on aPKC as we don't see a complete disruption or loss of ezrin abundance and phosphorylation at the apical surface in the absence of aPKC kinase activity. As mentioned in the introduction above, several kinases have been shown to phosphorylate ezrin in different systems and (7–11). Thus, there is a possibility of other kinases present in the ST regulate ezrin localization and phosphorylation in the absence of aPKCs. However, our study strongly indicates that aPKC kinase activity is important for the apical regulation of ezrin phosphorylation and abundance.

Importantly, our data reveal a profound regional decrease (~50% decrease) in the apical F-actin abundance in the absence of aPKC expression and function. These findings were consistent in both the first trimester (9-12 weeks) and term (37-40 weeks) placental ST implicating a strong role of the polarity regulating aPKC kinase in maintaining F-actin abundance. Interestingly, the decrease in F-actin abundance at the ST apical surface was regionalized, that is, the decrease in phalloidin intensity was a combination of areas with no visible decrease in the phalloidin signal intensity interspersed with the areas with significantly decreased F-actin. As observed in TE determination during human embryogenesis Par complex and F-actin have an interesting polarity dynamic where aPKCs and Par-6 follow the apical accumulation of F-actin (30). Thus, our results along with this study showcase the complex dynamics between polarity regulating Par complex and F-actin. Our data also implicates the regionalized effect of aPKCs at the ST apical surface. Thus, further studies addressing this region-specific effect of aPKCs will be very intriguing. Studies that not only look at the spatial specificity of aPKCs, but also temporal effects of aPKC at various stages in gestation will be interesting to understand the dynamic polarization that happens at the ST level.

Taken together, our data suggests that aPKC kinase activity not only regulates the F-actin anchoring protein – ezrin but it also maintains the F-actin abundance. These implicate a crucial role of aPKCs in maintaining the ST ezrin and F-actin abundance. However, one of the limitations of our study is that we did not look whether these interactions between aPKC, ezrin and F-actin are directly linked to each other or whether other proteins are responsible for this complex yet interesting dynamic in this highly polarized and critical ST epithelium. Thus, further studies are warranted in this novel field of studying polarity and actin dynamics in the ST.

It is critical to note that siRNA knockdown of either of the aPKC isoforms also led to the loss of F-actin, similar to what was observed with aPKC inhibitor treatment. Taken together, these data suggest that both, the expression as well as the function of aPKCs are critical in regulating ST F-actin dynamics. The knockdown data also shed light on the functional redundancy of aPKCs in regulating F-actin for loss of either of the isoforms significantly decreased apical F-actin abundance, with no additive effect observed when both the isoforms were absent (Figure 4.9 A-B). However, whether the lack of additive effect on F-actin abundance in the case of double isoform specific siRNA knockdown is a result of one of the limitations of our explant model where loss of one aPKC isoform might also decrease its scaffolding partners like Par 6, for the other isoform to bind to it, would be an interesting area to investigate.

With the visible loss of F-actin abundance, we anticipated severe apical surface changes. As mentioned in the results above, our EM images revealed a regionalized loss of microvilli on the ST apical surface. As expected, we noticed a severely altered ST apical surface. In addition to the coalition of the few remaining microvilli, the overall surface of the ST had a very porous appearance, which could almost be described as a lace-like surface. Our data also shows a profound change in the ST permeability to a 10,000MW neutral compound with reduced aPKC function or expression. It is known that dextran is taken up via macropinocytosis - a regulated form of endocytosis. This process of macropinocytosis is in turn an actin-dependent process (31). It is important to note that the increased sum of dextran signal was restricted to phalloidin-devoid areas suggesting that the loss of apical F-actin regionally coincides with the increased dextran uptake. Thus, further studies looking at the chronology of these functional disruptions are required

to better understand the underlying mechanisms of the association between the loss of polarity complex, loss of apical F-actin and the permeabilization of the ST.

We also determined the release of the hormone hCG-β in the absence of aPKC kinase activity, we saw a 3-fold increase in the extracellular release of hCG-β in explant conditioned medium of first trimester explants. The porous appearance of ST apical surface in SEM images of aPKC kinase inhibitor-treated explants suggests that this may be due to the bulk release of cytoplasmic contents, however, further experiments are required to confirm if release is due to exocytosis or cytoplasmic leakage.

Overall, our data reveals that decreased aPKC kinase activity can alter actin-linker protein ezrin phosphorylation and abundance as well as altered F-actin abundance at the ST apical surface.

Our EM images not only reveals the loss of ST apical microvilli, but it also exposes the formation of membrane pores at the ST surface when aPKC kinase activity is inhibited. While altered levels of ezrin are linked to various pathologies in different systems (4–6), decreased actin levels have been associated with depolarization, altered cell shape and release of mitochondrial reactive oxygen species (ROS) that ultimately leads to cell death in yeast and human epithelial cells (32,33). Additionally, formation of membrane pores has recently been linked with cell death pathways like necroptosis and pyroptosis. Necroptosis and pyroptosis are considered major types of programmed pro-inflammatory cell death (34). The underlying molecular mechanisms for both necroptosis and pyroptosis have only been recently determined, and distinct inflammatory cascades involving activation of mixed lineage kinase domain-like protein (MLKL) and gasdermin D (GSDMD), respectively have been elucidated (35). Studies regarding these pathways in the

pathogenesis of placental dysfunction have been very limited, but it has been recently shown that GSDMD is upregulated in preterm placentas with preeclampsia in comparison to preterm controls. Furthermore, the production of both interleukin-1β and interleukin-18, which are also markers for pyroptosis, have also been shown to be increased in preeclamptic placentas (35). Critically, our EM images showing microvilli replete surfaces were similar to the ultra-micrographs of the placentas obtained from pregnancy complications such as preeclampsia (36). As such, pyroptosis may be a critical inflammatory cell death pathway in the placental ST during preeclampsia pathogenesis. However, the exact cellular mechanisms, especially those associated with loss of polarity potentially initiating the pyroptotic cell-death program in the placenta have not been addressed. Thus, future directions include examining whether loss of cell polarity regulating aPKCs can initiate pyroptosis in the ST.

4.5 References

1. Shaha S, Patel K, Saadat S, Panahi S, de Almeida MM, Voronova A, et al. Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c. Placenta. 2022 Mar;119:39–43.

2. Berryman M, Gary R, Bretscher A. Ezrin oligomers are major cytoskeletal components of placental microvilli: a proposal for their involvement in cortical morphogenesis. J Cell Biol. 1995 Dec 1;131(5):1231–42.

3. Bretscher A, Reczek D, Berryman M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. J Cell Sci. 1997 Dec 15;110(24):3011–8.

4. Barik GK, Sahay O, Paul D, Santra MK. Ezrin gone rogue in cancer progression and metastasis: An enticing therapeutic target. Biochim Biophys Acta BBA - Rev Cancer. 2022 Jul;1877(4):188753.

5. Cheng Cy, Gungor-Ordueri Ne, Celik-Ozenci C. Ezrin: a regulator of actin microfilaments in cell junctions of the rat testis. Asian J Androl. 2015;17(4):653.

6. Nishimura T, Higuchi K, Sai Y, Sugita Y, Yoshida Y, Tomi M, et al. Fetal growth retardation and lack of hypotaurine in ezrin knockout mice. PloS One. 2014;9(8):e105423.

7. ten Klooster JP, Jansen M, Yuan J, Oorschot V, Begthel H, Di Giacomo V, et al. Mst4 and Ezrin Induce Brush Borders Downstream of the Lkb1/Strad/Mo25 Polarization Complex. Dev Cell. 2009 Apr;16(4):551–62.

8. Ng T. Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. EMBO J. 2001 Jun 1;20(11):2723–41.

9. Stapleton G, Malliri A, Ozanne BW. Downregulated AP-1 activity is associated with inhibition of Protein-Kinase-C-dependent CD44 and ezrin localisation and upregulation of PKC theta in A431 cells. J Cell Sci. 2002 Jul 1;115(13):2713–24.

10. Shiue H, Musch MW, Wang Y, Chang EB, Turner JR. Akt2 Phosphorylates Ezrin to Trigger NHE3 Translocation and Activation. J Biol Chem. 2005 Jan;280(2):1688–95.

11. Li X, Leu S, Cheong A, Zhang H, Baibakov B, Shih C, et al. Akt2, phosphatidylinositol 3-kinase, and PTEN are in lipid rafts of intestinal cells: Role in absorption and differentiation. Gastroenterology. 2004 Jan;126(1):122–35.

12. Ahuja V, Dieckgraefe BK, Anant S. Molecular biology of the small intestine: Curr Opin Gastroenterol. 2006 Mar;22(2):90–4.

13. Wald FA, Oriolo AS, Mashukova A, Fregien NL, Langshaw AH, Salas PJI. Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. J Cell Sci. 2008 Mar 1;121(5):644–54.

14. Pearson RB, Kemp BE. [3] Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. In: Methods in Enzymology [Internet]. Elsevier; 1991 [cited 2022 Aug 14]. p. 62–81. Available from: https://linkinghub.elsevier.com/retrieve/pii/007668799100127I

15. Fouassier L, Nichols MT, Gidey E, McWilliams RR, Robin H, Finnigan C, et al. Protein kinase C regulates the phosphorylation and oligomerization of ERM binding phosphoprotein 50. Exp Cell Res. 2005 May;306(1):264–73.

16. Miura S, Sato K, Kato-Negishi M, Teshima T, Takeuchi S. Fluid shear triggers microvilli formation via mechanosensitive activation of TRPV6. Nat Commun. 2015 Dec;6(1):8871.

17. Pollard TD, Cooper JA. Actin, a Central Player in Cell Shape and Movement. Science. 2009 Nov 27;326(5957):1208–12.

18. Schreiner T, Mohrs MR, Blau-Wasser R, von Krempelhuber A, Steinert M, Schleicher M, et al. Loss of the F-Actin Binding and Vesicle-Associated Protein Comitin Leads to a Phagocytosis Defect. Eukaryot Cell. 2002 Dec;1(6):906–14.

19. Sanjari Moghaddam H, Aarabi MH. Aβ-Mediated Dysregulation of F-Actin Nanoarchitecture Leads to Loss of Dendritic Spines and Alzheimer's Disease-Related Cognitive Impairments. J Neurosci. 2018 Jun 27;38(26):5840–2.

20. Ren W, Zhao W, Cao L, Huang J. Involvement of the Actin Machinery in Programmed Cell Death. Front Cell Dev Biol. 2021 Feb 9;8:634849.

21. Überall F, Hellbert K, Kampfer S, Maly K, Villunger A, Spitaler M, et al. Evidence That Atypical Protein Kinase C-λ and Atypical Protein Kinase C-ζ Participate in Ras-mediated Reorganization of the F-actin Cytoskeleton. J Cell Biol. 1999 Feb 8;144(3):413–25.

22. Patel K, Nguyen J, Shaha S, Zubkowski A, Riddell M. Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface [Internet]. Developmental Biology; 2022 Jul [cited 2022 Oct 13]. Available from: http://biorxiv.org/lookup/doi/10.1101/2022.06.30.498172

23. Forbes K, Desforges M, Garside R, Aplin JD, Westwood M. Methods for siRNA-mediated Reduction of mRNA and Protein Expression in Human Placental Explants, Isolated Primary Cells and Cell Lines. Placenta. 2009 Feb;30(2):124–9.

24. Whyte A. Lectin binding by microvillous membranes and coated-pit regions of human syncytial trophoblast. Histochem J. 1980 Sep;12(5):599–607.

25. Sivasubramaniyam T, Garcia J, Tagliaferro A, Melland-Smith M, Chauvin S, Post M, et al. Where Polarity Meets Fusion: Role of Par6 in Trophoblast Differentiation during Placental Development and Preeclampsia. Endocrinology. 2013 Mar 1;154(3):1296–309.

26. Grune T, Reinheckel T, North JA, Li R, Bescos PB, Shringarpure R, et al. Ezrin turnover and cell shape changes catalyzed by proteasome in oxidatively stressed cells. FASEB J. 2002 Oct;16(12):1602–10.

27. Georgiou M, Marinari E, Burden J, Baum B. Cdc42, Par6, and aPKC Regulate Arp2/3-Mediated Endocytosis to Control Local Adherens Junction Stability. Curr Biol. 2008 Nov;18(21):1631–8.

28. Ebnet K, Gerke V. Rho and Rab Family Small GTPases in the Regulation of Membrane Polarity in Epithelial Cells. Front Cell Dev Biol. 2022 Jul 4;10:948013.

29. Etienne-Manneville S, Hall A. Integrin-Mediated Activation of Cdc42 Controls Cell Polarity in Migrating Astrocytes through PKCζ. Cell. 2001 Aug;106(4):489–98.

30. Zhu M, Shahbazi M, Martin A, Zhang C, Sozen B, Borsos M, et al. Human embryo polarization requires PLC signaling to mediate trophectoderm specification. eLife. 2021 Sep 27;10:e65068.

31. Canton J. Macropinocytosis: New Insights Into Its Underappreciated Role in Innate Immune Cell Surveillance. Front Immunol. 2018 Oct 2;9:2286.

32. Gourlay CW, Carpp LN, Timpson P, Winder SJ, Ayscough KR. A role for the actin cytoskeleton in cell death and aging in yeast. J Cell Biol. 2004 Mar 15;164(6):803–9.

33. White SR, Williams P, Wojcik KR, Sun S, Hiemstra PS, Rabe KF, et al. Initiation of Apoptosis by Actin Cytoskeletal Derangement in Human Airway Epithelial Cells. Am J Respir Cell Mol Biol. 2001 Mar 1;24(3):282–94.

34. Bertheloot D, Latz E, Franklin BS. Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. Cell Mol Immunol. 2021 May;18(5):1106–21.

35. Cheng SB, Nakashima A, Huber WJ, Davis S, Banerjee S, Huang Z, et al. Pyroptosis is a critical inflammatory pathway in the placenta from early onset preeclampsia and in human trophoblasts exposed to hypoxia and endoplasmic reticulum stressors. Cell Death Dis. 2019 Dec;10(12):927.

36. Jones CJP, Fox H. An ultrastructural and ultrahistochemical study of the placenta of the diabetic woman. J Pathol. 1976 Jun;119(2):91–9.

Chapter 5 – Discussion

5.1 Summary of the key findings

In Chapter 3, we found that multiple isoforms of aPKC are expressed in the human placenta. Our western blot analysis reveals the protein expression of full length aPKC isoforms – aPKC-ι and aPKC-ζ. Additionally, immunoblotting with anti- aPKC-ζ led to a second prominent band near 55kDa. From the previous literature, we know that there is a brain specific N-terminally truncated aPKC-ζ isoform – PKM-ζ around ~50kDa (1). It is a constitutively active isoform due to the absence of the PSR that holds the full length aPKC isoforms in auto-inhibited state by binding to its kinase domain (1). Even though aPKC-ζ and PKM-ζ are the only known aPKC-ζ isoforms expressed at the protein level in the humans, various mRNA products and their predicted proteins have been annotated from the *PRKCZ* gene. Hence, we performed RT-PCR to examine which *PRKCZ* mRNA isoforms may be expressed in the placenta and trophoblasts. Interestingly, primers targeting *PRKCZ* isoform 3 which would encode a protein that lacks the N-terminal PB-1 domain and has a predicted molecular weight of 56 kDa successfully amplified products from the human placenta and trophoblasts in addition to primers for full length PRKCZ isoform. Thus, our study established the expression of two aPKC-ζ isoforms – the full length aPKC-ζ and N-terminally truncated aPKC-ζ III isoform along with the full length aPKC-ι, in human placenta and trophoblasts (Fig 3.8). We also established that three isoforms of aPKC - aPKC-ι, aPKC-ζ and aPKC-ζ III are expressed in vCTs and the highly polarized ST.

In chapter 4 of this thesis, we focused on understanding how aPKC isoforms expressed in the human placenta may regulate ST polarity. In the first half of chapter 4, we provide evidence of how aPKC-ι and aPKC-ζ isoforms colocalize with the cytoskeletal linker protein ezrin at the ST apical membrane. This colocalization significantly increased with increase in gestational age and was similar during the late first trimester as well as term (Fig 4.1 and 4.2). Next, our data reveals

the critical role aPKC kinase plays in regulating ezrin abundance and phosphorylation at the ST apical surface in both late first trimester and term placental explants (Fig 4.3 and 4.4). We also show that inhibiting total aPKC kinase activity leads to a significant decrease in apical F-actin in late first trimester as well as term explants (Fig 4.5). Due to the presence of multiple aPKC isoforms in the human placenta, and the known compensatory nature of aPKCs, we performed isoform specific knockdown of aPKCs to investigate whether the loss of a single aPKC isoform can compensate for the other. Interestingly, our data rather revealed the functionally redundant nature of aPKCs in the human ST, as loss of either of the isoforms led to a significant decrease in apical F-actin (Fig 4.9 and 4.10).

In the second half of chapter 4, we extended this study and found that inhibiting total aPKC kinase activity not only led to a significant decrease F-actin, but also the F-actin rich microvilli abundance. Our SEM images reveal a region-specific loss of apical microvilli as well as a porous appearance of the placental surface (Fig 4.11). Due to its presence at the maternal-fetal interface, the ST is known to play a critical role in the uptake of biomolecules and secretion of hormones amongst the other functions it performs. Thus, when we investigated whether this loss of apical microvilli and the porous surface occurred along with any functional alterations, we found that loss of aPKC activity led to a significantly increased dextran uptake as well as a significantly increased extracellular release of hCG-β (Fig 4.13 and 4.15). Thus, aPKC isoforms seem to play a pivotal role not only maintaining the ST apical surface but also the implicated functions of the ST.

In conclusion, we found that the human placenta expresses three aPKC isoforms: aPKC-ι, aPKC-ζ and aPKC-ζ III. The functional implications of their expression and kinase activity include the regulation of ST apical ezrin and F-actin, as well as the actin rich microvilli. As often stated in the previous chapters, microvilli act as a hallmark for established polarity in epithelial cells and

dynamic regulation of polarity is critical to maintain cellular homeostasis. Thus, our study establishes a key role of polarity regulating aPKC isoforms in maintaining the ST apical surface.

5.2 Overall significance

As mentioned in section 1.3, when the vCTs differentiate to form the ST, *in vitro* observations suggest that there are certain morphological and biochemical polarization of actin cytoskeleton and certain biomolecules, respectively (2,3). This asymmetric distribution of biomolecules at the apical membrane of the ST strongly supports the decades old literature on the compartmentalization of ST structure and functions (4). Despite the critical role ST plays in carrying out the placental functions, there is a huge gap in literature examining how polarity (which is synonymous to the structural compartmentalization) is maintained in the ST.

We know now that aPKCs are important regulators of polarity in the ST. Altered levels of aPKC or mislocalization of aPKC isoforms are linked with irritable bowel syndrome and multiple types of cancer (5–8). Wald *et.al* have shown that aPKC-ι play an indispensable role in phosphorylating intestinal microvillar ezrin at Thr567 in CACO-2 cell line (9). Altered ezrin levels are linked with cancer metastasis and have also been shown to decrease microvillar abundance in various cell types (10,11). ST microvilli are functionally involved in increasing the surface area for transport and hormone secretion. Additionally, they are also involved in release of STBEVs containing bioactive molecules required for maternal-fetal communication (12,13). It is known that there is loss of microvilli at the ST apical surface in placental pathologies associated with pregnancy complications like Preeclampsia (PE). PE is the *de novo* onset of hypertension and proteinuria or multisystem end-organ failure after ~ 20 weeks of gestation (14). It is known to affect 2-8% of pregnancy worldwide (14). Yet no one has investigated whether this association between placental pathologies and loss of microvilli is a cause or consequence of pregnancy complications (15). Here, we show that aPKC isoforms are critical for regulating the structure, functions, and

permeability of the ST apical surface. Hence our study suggests that disruption of ST polarity via altered expression or localization of aPKCs could be induced in the pathogenesis of PE.

5.3 Limitations

While our aim was to bridge the gap in knowledge about ST polarity, one of the overall caveats of our study is that we looked at a very narrow aspect of polarity. We looked at the aspects of polarity in terms of apical localization of ezrin and its phosphorylation, abundance of actin polarized and reorganized into microvilli. Additionally, using a placental explant model had its own strengths and limitations. One of the limitations of our explant model is that it is devoid of flow. Miura *et.al* have shown a critical role of fluid shear stress on the induction of microvilli formation in trophoblastic cells (16). Even though, our model lacked the external mechanosensitive cue, the presence of an intact ST, its villus branching and its microvilli, our model closely represented the physiological system. Another shortcoming was that initially we focused on the entire mounted sample, however, with time our focus of interest narrowed down to the terminal villi projections for all the imaging and quantification performed. To re-iterate the importance of terminal villi, these are the most differentiated villus projection and have a very thin layer of basement membrane between the ST and the blood capillaries, hence they have an enhanced diffusive exchange rate and most of the nutrient uptake and hormone secretion occurs here (See section 1.2 and Fig 1.2). Imaging the entire area of mounted samples would have provided us with much more interesting details, however for better time and resource management we limited our study to the terminal villi.

Notably, in chapter 3, one of the limitations was the lack of an antibody specific for the aPKC-ζ III isoform. However, our siRNA knockdown validated the antibody that was used to be specific for both aPKC-ζ isoforms.

In chapter 4, the antibody that we used for staining phospho-ezrin (Thr567) is specific for all the ERM proteins. However, previous literature and human protein atlas has found that the placenta most abundantly express ezrin and very little or negligible expression of radixin and moesin is found at the ST apical surface. While we wanted to study the effect of aPKC inhibition on ezrin phosphorylation and F-actin together to understand the direct link between their altered expression levels, we were unable to do so, as the ezrin specific antibodies required antigen retrieval and the phalloidin antibody that stained for F-actin did not work with antigen retrieval. Thus, we were unable to address the direct link between the effect of aPKC inhibition on the cytoskeleton and the cytoskeletal linker protein.

Additionally, the myristoylated aPKC pseudosubstrate inhibitor used in chapter 4 for various experiments has a few limitations of its own. It is a PKC-ζ inhibitor, however both PKCλ/ι and PKCζ are known to have identical pseudo-substrates hence this inhibitor targets both the aPKCs. The effective interaction of the inhibitor with half-maximal (IC_{50}) concentration has been found to be around 4.6μM (17). However, at higher concentrations it has been shown to have off-target effects and inhibit various PKC isoforms(17). The myristoylation process is a lipid modification where the myristoyl group derived from myristic acid covalently binds to the alpha-amino group of the N-terminal glycine residue of the protein. However, increased myristoylation process has been linked with carcinogenesis and developmental disorders (18–20). Hence, there is a possibility that the myristoylation itself could adversely affect the explant culture.

Thus, to rule out the off-target effects of the aPKC inhibitor, we also performed isoform specific siRNA knockdown. Our data reveals identical outcomes when explants are treated with aPKC inhibitor and siRNA knockdown, thus ruling out the possibility of off-target effects.

5.4 Future Directions

5.4.1 APKC-Actin Pathway

Our study reveals the expanding role aPKCs play in maintaining the various aspects of ST polarity including the regulation of apical F-actin. In *Drosophila*, post-synaptic F-actin organization in neuromuscular junction was indirectly regulated by aPKC mediated phosphorylation of Baz (Par-3 in humans) (21). Another polarity study in *Drosophila* neuroblast revealed that actin cytoskeleton is required for the retention of aPKC at the apical domain (22). However, in different cell systems, the pathway interlinking aPKCs and actin is not clear. Thus, it would be reasonable to expand our knowledge in understanding whether aPKC works upstream or downstream of actin cytoskeleton in the human ST.

To address this, we can use the same explant model and treat them with Latrunculin A (LatA). LatA binds to actin monomers and prevents them from polymerizing. In our study, the minimal time frame for which we treated the explants with aPKC inhibitor before monitoring the F-actin abundance is 2 hours and we saw a significant loss of F-actin abundance (see Fig 4.5). Actin treadmilling is a complex process involving its interaction with various other proteins such as Formin, Cofilin, Arp2/3, thus F-actin turnover is a very subjective process dependent on growth depletion feedback mechanism. However, its turnover is in the range of a few seconds. (23). We can design an experiment where we treat the placental explants with LatA for different time points starting with 0.5h, 1h, 1.5h and 2h and then perform IHC using aPKC antibodies and investigate whether the loss of F-actin can alter aPKC expression levels. If we do not observe a significant loss of aPKC isoforms at the ST apical membrane, it would suggest that aPKCs work upstream of F-actin.

Another experiment that we can perform to confirm whether aPKC kinase activity directly regulates F-actin is to perform a rescue experiment. For this, we can perform aPKC inhibition as it is in chapter 4, however following the inhibition, we can perform a plasmid mediated overexpression of specific aPKC isoforms. However, for overexpression we will have to optimize a cell line model which is polarized enough to observe the implicated functions. To achieve this, we can isolate primary human trophoblasts and culture them in Transwell© with Matrigel to get polarized ST monolayer(24). Post this, we can perform similar set of functional experiments where we look at dextran uptake and extracellular release of hCG-β. If aPKC overexpression can prevent excessive dextran uptake and increased hCG-β release, it will be potentially clear that aPKCs regulate F-actin and its associated functions. Additionally, performing isoform specific overexpression will help us further confirm whether aPKCs are functionally redundant in maintaining ST F-actin abundance and related functions.

5.4.2 Is polarity loss pathologically relevant?

After establishing a clear pathway link between aPKCs and F-actin, the next step would be to understand whether this loss of polarity is linked with the established loss of microvilli in placentas from patients with PE. In addition to the known shedding of microvilli in maternal circulation electron micrographs of PE placenta reveal loss of ST apical microvilli and distorted actin cytoskeleton. These observations are similar to what we observed in our SEM images (Fig 4.11). Studies show the that mRNA of various transporters for amino acids such as Leucine (SLC3A2, SLC7A5), for iron (Tfr) and cholesterol (ABCA1, ABCG1) are expressed in the ST and most of these transporters are abundantly found in the ST microvillar region (25,26). With the evident loss of microvilli in the absence of aPKCs, it is only reasonable to hypothesize that these apically located placental transporters are also lost or mislocalized. Huang *et al* reveal a significant

decrease in the expression of cholesterol transporter ABCA1 and a significant increase in amino acid transporter SLC7A7 in the plasma membranes extracted from PE placentas (27). Impairment of ABCA1 led to compromised cholesterol and phospholipid efflux resulting in concomitant lipid accumulation in PE placentas(27,28). Additionally, SLC7A7 is a known transporter for amino acids such as lysine and arginine present in the microvillar membrane of the placenta. Increased expression of these transporters in PE placentas could potentially to reduced maternal lysine and arginine levels (27). For this we could perform bioinformatic analysis using microarray datasets available publicly and narrow down a few membrane transporters that are expressed at the ST plasma membrane. Following this we can do a comparative study between healthy term control vs. PE term vs. healthy term treated with aPKC inhibitor. Following this, we can do a biochemical quantification of mRNA and protein expression of the selected membrane transporters and explore whether there is a significant similarity in the altered membrane transporter expressions between the PE samples and term samples where aPKC expression is inhibited. For isoform specific effect on placental transporters, we can also perform siRNA knockdown of aPKCs instead of total aPKC inhibition. Thus, this will help us understand whether the loss of polarity regulating aPKCs impact the placental transporters in a way where it can contribute to placental pathologies.

Overall, our data extrapolates the critical role aPKCs play in regulating human ST polarity. Importantly, the data presented in this thesis lays a strong foundation for future studies examining the relevance of ST polarity dysregulation in pathological conditions such as PE.

5.5 References

1. Yao Y, Kelly MT, Sajikumar S, Serrano P, Tian D, Bergold PJ, et al. PKM Maintains Late Long-Term Potentiation by N-Ethylmaleimide-Sensitive Factor/GluR2-Dependent Trafficking of Postsynaptic AMPA Receptors. J Neurosci. 2008 Jul 30;28(31):7820–7.

2. Jaremek A, Jeyarajah MJ, Jaju Bhattad G, Renaud SJ. Omics Approaches to Study Formation and Function of Human Placental Syncytiotrophoblast. Front Cell Dev Biol. 2021 Jun 15;9:674162.

3. Renaud SJ, Jeyarajah MJ. How trophoblasts fuse: an in-depth look into placental syncytiotrophoblast formation. Cell Mol Life Sci. 2022 Aug;79(8):433.

4. Dempsey EW, Luse SA. Regional specializations in the syncytial trophoblast of early human placentas. J Anat. 1971 Apr;108(Pt 3):545–61.

5. Fields AP, Gustafson WC. Protein Kinase C in Disease: Cancer. In: Protein Kinase C Protocols [Internet]. New Jersey: Humana Press; 2003 [cited 2022 Sep 8]. p. 519–38. Available from: http://link.springer.com/10.1385/1-59259-397-6:519

6. Wald FA, Forteza R, Diwadkar-Watkins R, Mashukova A, Duncan R, Abreu MT, et al. Aberrant expression of the polarity complex atypical PKC and non-muscle myosin IIA in active and inactive inflammatory bowel disease. Virchows Arch Int J Pathol. 2011 Sep;459(3):331–8.

7. Mashukova A, Wald FA, Salas PJ. Tumor necrosis factor alpha and inflammation disrupt the polarity complex in intestinal epithelial cells by a posttranslational mechanism. Mol Cell Biol. 2011 Feb;31(4):756–65.

8. Kravtsov D, Mashukova A, Forteza R, Rodriguez MM, Ameen NA, Salas PJ. Myosin 5b loss of function leads to defects in polarized signaling: implication for microvillus inclusion disease pathogenesis and treatment. Am J Physiol-Gastrointest Liver Physiol. 2014 Nov 15;307(10):G992–1001.

9. Wald FA, Oriolo AS, Mashukova A, Fregien NL, Langshaw AH, Salas PJI. Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. J Cell Sci. 2008 Mar 1;121(5):644–54.

10. Barik GK, Sahay O, Paul D, Santra MK. Ezrin gone rogue in cancer progression and metastasis: An enticing therapeutic target. Biochim Biophys Acta BBA - Rev Cancer. 2022 Jul;1877(4):188753.

11. Bonilha VL, Finnemann SC, Rodriguez-Boulan E. Ezrin Promotes Morphogenesis of Apical Microvilli and Basal Infoldings in Retinal Pigment Epithelium. J Cell Biol. 1999 Dec 27;147(7):1533–48.

12. Davies R, Griffiths C, Askelund K, Palaiologou E, Cleal JK, Page A, et al. Microvillous tip vesicles may be an origin of placental extracellular vesicles. Placenta. 2022 Jun;123:24–30.

13. Tannetta D, Collett G, Vatish M, Redman C, Sargent I. Syncytiotrophoblast extracellular vesicles – Circulating biopsies reflecting placental health. Placenta. 2017 Apr;52:134–8.

14. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. Obstet Gynecol. 2020 Jun;135(6):e237–60.

15. Knight M, Redman CWG, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. BJOG Int J Obstet Gynaecol. 1998 Jun;105(6):632–40.

16. Miura S, Sato K, Kato-Negishi M, Teshima T, Takeuchi S. Fluid shear triggers microvilli formation via mechanosensitive activation of TRPV6. Nat Commun. 2015 Dec;6(1):8871.

17. Bogard AS, Tavalin SJ. Protein Kinase C (PKC) ζ Pseudosubstrate Inhibitor Peptide Promiscuously Binds PKC Family Isoforms and Disrupts Conventional PKC Targeting and Translocation. Mol Pharmacol. 2015 Oct;88(4):728–35.

18. Magnuson BA, Raju RVS, Moyana TN, Sharma RK. Increased N-Myristoyltransferase Activity Observed in Rat and Human Colonic Tumors. JNCI J Natl Cancer Inst. 1995 Nov 1;87(21):1630–5.

19. Raju RVS, Moyana TN, Sharma RK. N-Myristoyltransferase Overexpression in Human Colorectal Adenocarcinomas. Exp Cell Res. 1997 Aug;235(1):145–54.

20. Cordeddu V, Di Schiavi E, Pennacchio LA, Ma'ayan A, Sarkozy A, Fodale V, et al. Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. Nat Genet. 2009 Sep;41(9):1022–6.

21. Ramachandran P, Barria R, Ashley J, Budnik V. A critical step for postsynaptic F-actin organization: Regulation of Baz/Par-3 localization by aPKC and PTEN. Dev Neurobiol. 2009 Aug;69(9):583–602.

22. Oon CH, Prehoda KE. Asymmetric recruitment and actin-dependent cortical flows drive the neuroblast polarity cycle. eLife. 2019 May 8;8:e45815.

23. Bleicher P, Sciortino A, Bausch AR. The dynamics of actin network turnover is self-organized by a growth-depletion feedback. Sci Rep. 2020 Dec;10(1):6215.

24. Fuenzalida B, Kallol S, Lüthi M, Albrecht C, Leiva A. The polarized localization of lipoprotein receptors and cholesterol transporters in the syncytiotrophoblast of the placenta is reproducible in a monolayer of primary human trophoblasts. Placenta. 2021 Feb;105:50–60.

25. Karahoda R, Zaugg J, Fuenzalida B, Kallol S, Moser-Haessig R, Staud F, et al. Trophoblast Differentiation Affects Crucial Nutritive Functions of Placental Membrane Transporters. Front Cell Dev Biol. 2022 Feb 22;10:820286.

26. Walker N, Filis P, Soffientini U, Bellingham M, O'Shaughnessy PJ, Fowler PA. Placental transporter localization and expression in the Human: the importance of species, sex, and gestational age differences†. Biol Reprod. 2017 Apr 1;96(4):733–42.

27. Huang X, Anderle P, Hostettler L, Baumann MU, Surbek DV, Ontsouka EC, et al. Identification of placental nutrient transporters associated with intrauterine growth restriction and pre-eclampsia. BMC Genomics. 2018 Dec;19(1):173.

28. Huang X, Jain A, Baumann M, Körner M, Surbek D, Bütikofer P, et al. Increased Placental Phospholipid Levels in Pre-Eclamptic Pregnancies. Int J Mol Sci. 2013 Feb 6;14(2):3487–99.

Bibliography:

1. Azar C, Valentine M, Trausch-Azar J, Druley T, Nelson DM, Schwartz AL. RNA-Seq identifies genes whose proteins are transformative in the differentiation of cytotrophoblast to syncytiotrophoblast, in human primary villous and BeWo trophoblasts. Sci Rep. 2018 Dec;8(1):5142.

2. Shahbazi MN, Zernicka-Goetz M. Deconstructing and reconstructing the mouse and human early embryo. Nat Cell Biol. 2018 Aug;20(8):878–87.

3. Burton GJ, Jauniaux E. What is the placenta? Am J Obstet Gynecol. 2015 Oct;213(4 Suppl):S6.e1, S6-8.

4. Baergen RN, Burton GJ, Kaplan CG. Benirschke's Pathology of the Human Placenta. 2022.

5. Huppertz B. The anatomy of the normal placenta. J Clin Pathol. 2008 Dec;61(12):1296–302.

6. Benirschke K, Burton GJ, Baergen RN. Architecture of Normal Villous Trees. In: Benirschke K, Burton GJ, Baergen RN, editors. Pathology of the Human Placenta [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 101–44. Available from: https://doi.org/10.1007/978-3-642-23941-0_7

7. Baczyk D, Dunk C, Huppertz B, Maxwell C, Reister F, Giannoulias D, et al. Bi-potential behaviour of cytotrophoblasts in first trimester chorionic villi. Placenta. 2006 May;27(4–5):367–74.

8. Tarrade A, Lai Kuen R, Malassiné A, Tricottet V, Blain P, Vidaud M, et al. Characterization of Human Villous and Extravillous Trophoblasts Isolated from First Trimester Placenta. Lab Invest. 2001 Sep;81(9):1199–211.

9. Lager S, Powell TL. Regulation of Nutrient Transport across the Placenta. Journal of Pregnancy. 2012;2012:1–14.

10. Mori M, Ishikawa G, Luo SS, Mishima T, Goto T, Robinson JM, et al. The Cytotrophoblast Layer of Human Chorionic Villi Becomes Thinner but Maintains Its Structural Integrity During Gestation1. Biology of Reproduction. 2007 Jan 1;76(1):164–72.

11. Simpson RA, Mayhew TM, Barnes PR. From 13 weeks to term, the trophoblast of human placenta grows by the continuous recruitment of new proliferative units: a study of nuclear number using the disector. Placenta. 1992 Oct;13(5):501–12.

12. Danihel Ĺ, Gomolčák P, Korbel M, Pružinec J, Vojtaššák J, Janík P, et al. Expression of proliferation and apoptotic markers in human placenta during pregnancy. Acta Histochemica. 2002 Jan;104(4):335–8.

13. Dittmar T, Zänker KS, editors. Cell fusion in health and disease. Dordrecht: Springer; 2011. 2 p. (Advances in experimental medicine and biology).

14. Lu X, Wang R, Zhu C, Wang H, Lin HY, Gu Y, et al. Fine-Tuned and Cell-Cycle-Restricted Expression of Fusogenic Protein Syncytin-2 Maintains Functional Placental Syncytia. Cell Reports. 2017 Oct;21(5):1150–9.

15. Handwerger S. New insights into the regulation of human cytotrophoblast cell differentiation. Mol Cell Endocrinol. 2010 Jul 8;323(1):94–104.

16. Lavialle C, Cornelis G, Dupressoir A, Esnault C, Heidmann O, Vernochet C, et al. Paleovirology of ' *syncytins* ', retroviral *env* genes exapted for a role in placentation. Phil Trans R Soc B. 2013 Sep 19;368(1626):20120507.

17. Jaremek A, Jeyarajah MJ, Jaju Bhattad G, Renaud SJ. Omics Approaches to Study Formation and Function of Human Placental Syncytiotrophoblast. Front Cell Dev Biol. 2021 Jun 15;9:674162.

18. Teasdale F, Jean-Jacques G. Morphometric evaluation of the microvillous surface enlargement factor in the human placenta from mid-gestation to term. Placenta. 1985 Sep;6(5):375–81.

19. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine Regulation of Human Fetal Growth: The Role of the Mother, Placenta, and Fetus. Endocrine Reviews. 2006 Apr 1;27(2):141–69.

20. Kidima WB. Syncytiotrophoblast Functions and Fetal Growth Restriction during Placental Malaria: Updates and Implication for Future Interventions. BioMed Research International. 2015;2015:1–9.

21. Tannetta D, Masliukaite I, Vatish M, Redman C, Sargent I. Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia. Journal of Reproductive Immunology. 2017 Feb;119:98–106.

22. McConnell RE, Tyska MJ. Myosin-1a powers the sliding of apical membrane along microvillar actin bundles. Journal of Cell Biology. 2007 May 21;177(4):671–81.

23. McConnell RE, Higginbotham JN, Shifrin DA, Tabb DL, Coffey RJ, Tyska MJ. The enterocyte microvillus is a vesicle-generating organelle. Journal of Cell Biology. 2009 Jun 29;185(7):1285–98.

24. Danielsen EM, Hansen GH. Generation of Stable Lipid Raft Microdomains in the Enterocyte Brush Border by Selective Endocytic Removal of Non-Raft Membrane. Vinciguerra M, editor. PLoS ONE. 2013 Oct 4;8(10):e76661.

25. Sauvanet C, Wayt J, Pelaseyed T, Bretscher A. Structure, Regulation, and Functional Diversity of Microvilli on the Apical Domain of Epithelial Cells. Annu Rev Cell Dev Biol. 2015 Nov 13;31(1):593–621.

26. Viswanatha R, Bretscher A, Garbett D. Dynamics of ezrin and EBP50 in regulating microvilli on the apical aspect of epithelial cells. Biochem Soc Trans. 2014 Feb;42(1):189–94.

27. Viswanatha R, Ohouo PY, Smolka MB, Bretscher A. Local phosphocycling mediated by LOK/SLK restricts ezrin function to the apical aspect of epithelial cells. Journal of Cell Biology. 2012 Dec 10;199(6):969–84.

28. Turunen O, Wahlström T, Vaheri A. Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. J Cell Biol. 1994 Sep;126(6):1445–53.

29. Gary R, Bretscher A. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. MBoC. 1995 Aug;6(8):1061–75.

30. Reczek D, Bretscher A. The Carboxyl-terminal Region of EBP50 Binds to a Site in the Amino-terminal Domain of Ezrin That Is Masked in the Dormant Molecule. Journal of Biological Chemistry. 1998 Jul;273(29):18452–8.

31. Li Q, Nance MR, Kulikauskas R, Nyberg K, Fehon R, Karplus PA, et al. Self-masking in an Intact ERM-merlin Protein: An Active Role for the Central α-Helical Domain. Journal of Molecular Biology. 2007 Feb;365(5):1446–59.

32. Pearson MA, Reczek D, Bretscher A, Karplus PA. Structure of the ERM Protein Moesin Reveals the FERM Domain Fold Masked by an Extended Actin Binding Tail Domain. Cell. 2000 Apr;101(3):259–70.

33. Nishimura T, Higuchi K, Sai Y, Sugita Y, Yoshida Y, Tomi M, et al. Fetal growth retardation and lack of hypotaurine in ezrin knockout mice. PLoS One. 2014;9(8):e105423.

34. Margolis B, Borg JP. Apicobasal polarity complexes. Journal of Cell Science. 2005 Nov 15;118(22):5157–9.

35. Rodriguez-Boulan E, Macara IG. Organization and execution of the epithelial polarity programme. Nat Rev Mol Cell Biol. 2014 Apr;15(4):225–42.

36. Bornens M. Organelle positioning and cell polarity. Nat Rev Mol Cell Biol. 2008 Nov;9(11):874–86.

37. Zhu M, Shahbazi M, Martin A, Zhang C, Sozen B, Borsos M, et al. Human embryo polarization requires PLC signaling to mediate trophectoderm specification. Elife. 2021 Sep 27;10:e65068.

38. Gerri C, McCarthy A, Alanis-Lobato G, Demtschenko A, Bruneau A, Loubersac S, et al. Initiation of a conserved trophectoderm program in human, cow and mouse embryos. Nature. 2020 Nov 19;587(7834):443–7.

39. Chi F, Beniwal AS, Liu H. The apical domain defines the trophectoderm differentiation in early mammalian embryo by regulating YAP nuclear translocation. AME Med J. 2017 Oct;2:155–155.

40. Hong Y. aPKC: the Kinase that Phosphorylates Cell Polarity. F1000Res. 2018 Jun 25;7:903.

41. Hernandez AI, Blace N, Crary JF, Serrano PA, Leitges M, Libien JM, et al. Protein Kinase Mζ Synthesis from a Brain mRNA Encoding an Independent Protein Kinase Cζ Catalytic Domain. Journal of Biological Chemistry. 2003 Oct;278(41):40305–16.

42. Yao Y, Kelly MT, Sajikumar S, Serrano P, Tian D, Bergold PJ, et al. PKM Maintains Late Long-Term Potentiation by N-Ethylmaleimide-Sensitive Factor/GluR2-Dependent Trafficking of Postsynaptic AMPA Receptors. Journal of Neuroscience. 2008 Jul 30;28(31):7820–7.

43. Parker SS, Mandell EK, Hapak SM, Maskaykina IY, Kusne Y, Kim JY, et al. Competing molecular interactions of aPKC isoforms regulate neuronal polarity. Proc Natl Acad Sci USA. 2013 Aug 27;110(35):14450–5.

44. Tsokas P, Hsieh C, Yao Y, Lesburguères E, Wallace EJC, Tcherepanov A, et al. Compensation for PKMζ in long-term potentiation and spatial long-term memory in mutant mice. eLife. 2016 May 17;5:e14846.

45. Seidl S, Braun U, Roos N, Li S, Lüdtke THW, Kispert A, et al. Phenotypical Analysis of Atypical PKCs In Vivo Function Display a Compensatory System at Mouse Embryonic Day 7.5. Lim HJ, editor. PLoS ONE. 2013 May 14;8(5):e62756.

46. Soloff RS, Katayama C, Lin MY, Feramisco JR, Hedrick SM. Targeted Deletion of Protein Kinase C λ Reveals a Distribution of Functions between the Two Atypical Protein Kinase C Isoforms. J Immunol. 2004 Sep 1;173(5):3250–60.

47. Bhattacharya B, Home P, Ganguly A, Ray S, Ghosh A, Islam MdR, et al. Atypical protein kinase C iota (PKCλ/ι) ensures mammalian development by establishing the maternal–fetal exchange interface. Proc Natl Acad Sci USA. 2020 Jun 23;117(25):14280–91.

48. Visco I, Hoege C, Hyman AA, Schwille P. In vitro Reconstitution of a Membrane Switch Mechanism for the Polarity Protein LGL. Journal of Molecular Biology. 2016 Dec;428(24):4828–42.

49. Marikawa Y, Alarcon VB. Creation of Trophectoderm, the First Epithelium, in Mouse Preimplantation Development. In: Kubiak JZ, editor. Mouse Development [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012 [cited 2022 Aug 15]. p. 165–84. (Results and Problems in Cell Differentiation; vol. 55). Available from: http://link.springer.com/10.1007/978-3-642-30406-4_9

50. Nagai-Tamai Y, Mizuno K, Hirose T, Suzuki A, Ohno S. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells: Regulated interaction between aPKC and PAR-3. Genes to Cells. 2002 Nov;7(11):1161–71.

51. Holly RW, Jones K, Prehoda KE. A Conserved PDZ-Binding Motif in aPKC Interacts with Par-3 and Mediates Cortical Polarity. Current Biology. 2020 Mar;30(5):893-898.e5.

52. Plusa B, Piliszek A, Frankenberg S, Artus J, Hadjantonakis AK. Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. Development. 2008 Sep 15;135(18):3081–91.

53. Lopez-Garcia LA, Schulze JO, Fröhner W, Zhang H, Süß E, Weber N, et al. Allosteric Regulation of Protein Kinase PKCζ by the N-Terminal C1 Domain and Small Compounds to the PIF-Pocket. Chemistry & Biology. 2011 Nov;18(11):1463–73.

54. Shaha S, Patel K, Saadat S, Panahi S, de Almeida MM, Voronova A, et al. Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c. Placenta. 2022 Mar;119:39–43.

55. Berryman M, Gary R, Bretscher A. Ezrin oligomers are major cytoskeletal components of placental microvilli: a proposal for their involvement in cortical morphogenesis. Journal of Cell Biology. 1995 Dec 1;131(5):1231–42.

56. Bretscher A, Reczek D, Berryman M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. Journal of Cell Science. 1997 Dec 15;110(24):3011–8.

57. Barik GK, Sahay O, Paul D, Santra MK. Ezrin gone rogue in cancer progression and metastasis: An enticing therapeutic target. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2022 Jul;1877(4):188753.

58. Cheng Cy, Gungor-Ordueri Ne, Celik-Ozenci C. Ezrin: a regulator of actin microfilaments in cell junctions of the rat testis. Asian J Androl. 2015;17(4):653.

59. ten Klooster JP, Jansen M, Yuan J, Oorschot V, Begthel H, Di Giacomo V, et al. Mst4 and Ezrin Induce Brush Borders Downstream of the Lkb1/Strad/Mo25 Polarization Complex. Developmental Cell. 2009 Apr;16(4):551–62.

60. Viswanatha R, Bretscher A, Garbett D. Dynamics of ezrin and EBP50 in regulating microvilli on the apical aspect of epithelial cells. Biochemical Society Transactions. 2014 Feb 1;42(1):189–94.

61. Stapleton G, Malliri A, Ozanne BW. Downregulated AP-1 activity is associated with inhibition of Protein-Kinase-C-dependent CD44 and ezrin localisation and upregulation of PKC theta in A431 cells. Journal of Cell Science. 2002 Jul 1;115(13):2713–24.

62. Ng T. Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. The EMBO Journal. 2001 Jun 1;20(11):2723–41.

63. Shiue H, Musch MW, Wang Y, Chang EB, Turner JR. Akt2 Phosphorylates Ezrin to Trigger NHE3 Translocation and Activation. Journal of Biological Chemistry. 2005 Jan;280(2):1688–95.

64. Li X, Leu S, Cheong A, Zhang H, Baibakov B, Shih C, et al. Akt2, phosphatidylinositol 3-kinase, and PTEN are in lipid rafts of intestinal cells: Role in absorption and differentiation. Gastroenterology. 2004 Jan;126(1):122–35.

65. Wald FA, Oriolo AS, Mashukova A, Fregien NL, Langshaw AH, Salas PJI. Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. Journal of Cell Science. 2008 Mar 1;121(5):644–54.

66. Pearson RB, Kemp BE. [3] Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. In: Methods in Enzymology [Internet]. Elsevier; 1991 [cited 2022 Aug 15]. p. 62–81. Available from: https://linkinghub.elsevier.com/retrieve/pii/007668799100127I

67. Fouassier L, Nichols MT, Gidey E, McWilliams RR, Robin H, Finnigan C, et al. Protein kinase C regulates the phosphorylation and oligomerization of ERM binding phosphoprotein 50. Experimental Cell Research. 2005 May;306(1):264–73.

68. Miura S, Sato K, Kato-Negishi M, Teshima T, Takeuchi S. Fluid shear triggers microvilli formation via mechanosensitive activation of TRPV6. Nat Commun. 2015 Dec;6(1):8871.

69. Ahuja V, Dieckgraefe BK, Anant S. Molecular biology of the small intestine: Current Opinion in Gastroenterology. 2006 Mar;22(2):90–4.

70. Schreiner T, Mohrs MR, Blau-Wasser R, von Krempelhuber A, Steinert M, Schleicher M, et al. Loss of the F-Actin Binding and Vesicle-Associated Protein Comitin Leads to a Phagocytosis Defect. Eukaryot Cell. 2002 Dec;1(6):906–14.

71. Sanjari Moghaddam H, Aarabi MH. Aβ-Mediated Dysregulation of F-Actin Nanoarchitecture Leads to Loss of Dendritic Spines and Alzheimer's Disease-Related Cognitive Impairments. J Neurosci. 2018 Jun 27;38(26):5840–2.

72. Ren W, Zhao W, Cao L, Huang J. Involvement of the Actin Machinery in Programmed Cell Death. Front Cell Dev Biol. 2021 Feb 9;8:634849.

73. Pollard TD, Cooper JA. Actin, a Central Player in Cell Shape and Movement. Science. 2009 Nov 27;326(5957):1208–12.

74. Überall F, Hellbert K, Kampfer S, Maly K, Villunger A, Spitaler M, et al. Evidence That Atypical Protein Kinase C-λ and Atypical Protein Kinase C-ζ Participate in Ras-mediated Reorganization of the F-actin Cytoskeleton. Journal of Cell Biology. 1999 Feb 8;144(3):413–25.

75. Forbes K, Desforges M, Garside R, Aplin JD, Westwood M. Methods for siRNA-mediated Reduction of mRNA and Protein Expression in Human Placental Explants, Isolated Primary Cells and Cell Lines. Placenta. 2009 Feb;30(2):124–9.

76. Sivasubramaniyam T, Garcia J, Tagliaferro A, Melland-Smith M, Chauvin S, Post M, et al. Where Polarity Meets Fusion: Role of Par6 in Trophoblast Differentiation during Placental Development and Preeclampsia. Endocrinology. 2013 Mar 1;154(3):1296–309.

77. Grune T, Reinheckel T, North JA, Li R, Bescos PB, Shringarpure R, et al. Ezrin turnover and cell shape changes catalyzed by proteasome in oxidatively stressed cells. FASEB j. 2002 Oct;16(12):1602–10.

78. Gourlay CW, Carpp LN, Timpson P, Winder SJ, Ayscough KR. A role for the actin cytoskeleton in cell death and aging in yeast. Journal of Cell Biology. 2004 Mar 15;164(6):803–9.

79. White SR, Williams P, Wojcik KR, Sun S, Hiemstra PS, Rabe KF, et al. Initiation of Apoptosis by Actin Cytoskeletal Derangement in Human Airway Epithelial Cells. Am J Respir Cell Mol Biol. 2001 Mar 1;24(3):282–94.

80. Jones CJP, Fox H. An ultrastructural and ultrahistochemical study of the placenta of the diabetic woman. J Pathol. 1976 Jun;119(2):91–9.

81. Canton J. Macropinocytosis: New Insights Into Its Underappreciated Role in Innate Immune Cell Surveillance. Front Immunol. 2018 Oct 2;9:2286.

82. Bertheloot D, Latz E, Franklin BS. Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. Cell Mol Immunol. 2021 May;18(5):1106–21.

83. Cheng SB, Nakashima A, Huber WJ, Davis S, Banerjee S, Huang Z, et al. Pyroptosis is a critical inflammatory pathway in the placenta from early onset preeclampsia and in human trophoblasts exposed to hypoxia and endoplasmic reticulum stressors. Cell Death Dis. 2019 Dec;10(12):927.

84. Lash GE, Naruse K, Innes BA, Robson SC, Searle RF, Bulmer JN. Secretion of Angiogenic Growth Factors by Villous Cytotrophoblast and Extravillous Trophoblast in Early Human Pregnancy. Placenta. 2010 Jun;31(6):545–8.

85. Robbins JR, Bakardjiev AI. Pathogens and the placental fortress. Current Opinion in Microbiology. 2012 Feb;15(1):36–43.

86. Erlebacher A. Immunology of the Maternal-Fetal Interface. Annu Rev Immunol. 2013 Mar 21;31(1):387–411.

87. Zeldovich VB, Bakardjiev AI. Host Defense and Tolerance: Unique Challenges in the Placenta. Heitman J, editor. PLoS Pathogens. 2012 Aug 9;8(8):e1002804.

88. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site: Inflammation and pregnancy. Annals of the New York Academy of Sciences. 2011 Mar;1221(1):80–7.

89. Delorme-Axford E, Donker RB, Mouillet JF, Chu T, Bayer A, Ouyang Y, et al. Human placental trophoblasts confer viral resistance to recipient cells. Proc Natl Acad Sci USA. 2013 Jul 16;110(29):12048–53.

90. Robbins JR, Skrzypczynska KM, Zeldovich VB, Kapidzic M, Bakardjiev AI. Placental Syncytiotrophoblast Constitutes a Major Barrier to Vertical Transmission of Listeria monocytogenes. Schneider DS, editor. PLoS Pathog. 2010 Jan 22;6(1):e1000732.

91. Robbins JR, Zeldovich VB, Poukchanski A, Boothroyd JC, Bakardjiev AI. Tissue Barriers of the Human Placenta to Infection with Toxoplasma gondii. Adams JH, editor. Infect Immun. 2012 Jan;80(1):418–28.

92. Koi H, Zhang J, Makrigiannakis A, Getsios S, MacCalman CD, Strauss JF, et al. Syncytiotrophoblast Is a Barrier to Maternal-Fetal Transmission of Herpes Simplex Virus1. Biology of Reproduction. 2002 Nov 1;67(5):1572–9.

93. Mengaud J, Ohayon H, Gounon P, Mège RM, Cossart P. E-Cadherin Is the Receptor for Internalin, a Surface Protein Required for Entry of L. monocytogenes into Epithelial Cells. Cell. 1996 Mar;84(6):923–32.

94. Bonazzi M, Cossart P. Impenetrable barriers or entry portals? The role of cell–cell adhesion during infection. Journal of Cell Biology. 2011 Oct 31;195(3):349–58.

95. Zeldovich VB, Clausen CH, Bradford E, Fletcher DA, Maltepe E, Robbins JR, et al. Placental Syncytium Forms a Biophysical Barrier against Pathogen Invasion. Wessels MR, editor. PLoS Pathog. 2013 Dec 12;9(12):e1003821.

96. Costa MA. The endocrine function of human placenta: an overview. Reproductive BioMedicine Online. 2016 Jan;32(1):14–43.

97. Balkovetz DF, Tiruppathi C, Leibach FH, Mahesh VB, Ganapathy V. Evidence for an Imipramine-sensitive Serotonin Transporter in Human Placental Brush-border Membranes. Journal of Biological Chemistry. 1989 Feb;264(4):2195–8.

98. Illsley NP. CURRENT TOPIC: Glucose Transporters in the Human Placenta. Placenta. 2000 Jan;21(1):14–22.

99. Sata R, Ohtani H, Tsujimoto M, Murakami H, Koyabu N, Nakamura T, et al. Functional Analysis of Organic Cation Transporter 3 Expressed in Human Placenta. J Pharmacol Exp Ther. 2005 Nov;315(2):888–95.

100. Cleal JK, Lofthouse EM, Sengers BG, Lewis RM. A systems perspective on placental amino acid transport: A systems perspective on placental amino acid transport. J Physiol. 2018 Dec;596(23):5511–22.

101. Bloise E, Ortiga-Carvalho TM, Reis FM, Lye SJ, Gibb W, Matthews SG. ATP-binding cassette transporters in reproduction: a new frontier. Hum Reprod Update. 2015 Nov 5;dmv049.

102. Apáti Á, Szebényi K, Erdei Z, Várady G, Orbán TI, Sarkadi B. The importance of drug transporters in human pluripotent stem cells and in early tissue differentiation. Expert Opinion on Drug Metabolism & Toxicology. 2016 Jan 2;12(1):77–92.

103. Karahoda R, Zaugg J, Fuenzalida B, Kallol S, Moser-Haessig R, Staud F, et al. Trophoblast Differentiation Affects Crucial Nutritive Functions of Placental Membrane Transporters. Front Cell Dev Biol. 2022 Feb 22;10:820286.

104. Mayhew TM, Burton GJ. Stereology and its impact on our understanding of human placental functional morphology. Microsc Res Tech. 1997 Jul 1;38(1–2):195–205.

105. Huppertz B, Gauster M. Trophoblast Fusion. In: Dittmar T, Zänker KS, editors. Cell Fusion in Health and Disease [Internet]. Dordrecht: Springer Netherlands; 2011 [cited 2022 Aug 30]. p. 81–95. (Advances in Experimental Medicine and Biology; vol. 713). Available from: http://link.springer.com/10.1007/978-94-007-0763-4_6

106. Ishikawa A, Omata W, Ackerman WE, Takeshita T, Vandré DD, Robinson JM. Cell fusion mediates dramatic alterations in the actin cytoskeleton, focal adhesions, and E-cadherin in

trophoblastic cells: Trophoblast Fusion and Cellular Alterations. Cytoskeleton. 2014 Apr;71(4):241–56.

107. Ibrahim MEA, Al-Zuhair AGH, Mughal S, Hathout H. Surface ultrastructure of the human placental villi and sites of contact with maternal red blood cells. Archives of gynecology. 1982 Dec 1;233(1):67–72.

108. Tanentzapf G, Tepass U. Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. Nat Cell Biol. 2003 Jan;5(1):46–52.

109. Kemphues KJ, Priess JR, Morton DG, Cheng N. Identification of genes required for cytoplasmic localization in early C. elegans embryos. Cell. 1988 Feb;52(3):311–20.

110. Guo S, Kemphues KJ. A non-muscle myosin required for embryonic polarity in Caenorhabditis elegans. Nature. 1996 Aug;382(6590):455–8.

111. Watts JL, Etemad-Moghadam B, Guo S, Boyd L, Draper BW, Mello CC, et al. par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3. Development. 1996 Oct 1;122(10):3133–40.

112. Etemad-Moghadam B, Guo S, Kemphues KJ. Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early C. elegans embryos. Cell. 1995 Dec;83(5):743–52.

113. Izumi Y, Hirose T, Tamai Y, Hirai S ichi, Nagashima Y, Fujimoto T, et al. An Atypical PKC Directly Associates and Colocalizes at the Epithelial Tight Junction with ASIP, a Mammalian Homologue of Caenorhabditis elegans Polarity Protein PAR-3. Journal of Cell Biology. 1998 Oct 5;143(1):95–106.

114. Joberty G, Petersen C, Gao L, Macara IG. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol. 2000 Aug;2(8):531–9.

115. Harris TJC, Peifer M. The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. Journal of Cell Biology. 2005 Aug 29;170(5):813–23.

116. Suzuki A, Ishiyama C, Hashiba K, Shimizu M, Ebnet K, Ohno S. aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. Journal of Cell Science. 2002 Sep 15;115(18):3565–73.

117. Tabuse Y, Izumi Y, Piano F, Kemphues KJ, Miwa J, Ohno S. Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. Development. 1998 Sep 15;125(18):3607–14.

118. Tepass U. Crumbs, a Component of the Apical Membrane, Is Required for Zonula Adherens Formation in Primary Epithelia ofDrosophila. Developmental Biology. 1996 Jul;177(1):217–25.

119. Bazellieres E. Crumbs proteins in epithelial morphogenesis. Front Biosci. 2009;Volume(14):2149.

120. Wodarz A, Hinz U, Engelbert M, Knust E. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of drosophila. Cell. 1995 Jul;82(1):67–76.

121. Wodarz A, Grawe F, Knust E. CRUMBS is involved in the control of apical protein targeting during Drosophila epithelial development. Mechanisms of Development. 1993 Dec;44(2–3):175–87.

122. Klebes A, Knust E. A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in Drosophila. Current Biology. 2000 Jan;10(2):76–85.

123. Bachmann A, Schneider M, Theilenberg E, Grawe F, Knust E. Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. Nature. 2001 Dec 6;414(6864):638–43.

124. Katoh M, Katoh M. Identification and characterization of Crumbs homolog 2 gene at human chromosome 9q33.3. Int J Oncol. 2004 Mar;24(3):743–9.

125. Chartier FJM, Hardy ÉJL, Laprise P. Crumbs controls epithelial integrity by inhibiting Rac1 and PI3K. Journal of Cell Science. 2011 Oct 15;124(20):3393–8.

126. Dudok JJ, Murtaza M, Henrique Alves C, Rashbass P, Wijnholds J. Crumbs 2 prevents cortical abnormalities in mouse dorsal telencephalon. Neuroscience Research. 2016 Jul;108:12–23.

127. Roh MH, Makarova O, Liu CJ, Shin, Lee S, Laurinec S, et al. The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. Journal of Cell Biology. 2002 Apr 1;157(1):161–72.

128. Straight SW, Shin K, Fogg VC, Fan S, Liu CJ, Roh M, et al. Loss of PALS1 expression leads to tight junction and polarity defects. Mol Biol Cell. 2004 Apr;15(4):1981–90.

129. Lemmers C, Médina E, Delgrossi MH, Michel D, Arsanto JP, Le Bivic A. hINADl/PATJ, a Homolog of Discs Lost, Interacts with Crumbs and Localizes to Tight Junctions in Human Epithelial Cells. Journal of Biological Chemistry. 2002 Jul;277(28):25408–15.

130. Shin K, Wang Q, Margolis B. PATJ regulates directional migration of mammalian epithelial cells. EMBO Rep. 2007 Feb;8(2):158–64.

131. Bilder D, Perrimon N. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature. 2000 Feb;403(6770):676–80.

132. Benton R, Johnston DS. Drosophila PAR-1 and 14-3-3 Inhibit Bazooka/PAR-3 to Establish Complementary Cortical Domains in Polarized Cells. Cell. 2003 Dec;115(6):691–704.

133. Yamanaka T. Role of Lgl/Dlg/Scribble in the regulation of epithelial junction, polarity and growth. Front Biosci. 2008;Volume(13):6693.

134. Laprise P. Emerging Role for Epithelial Polarity Proteins of the Crumbs Family as Potential Tumor Suppressors. Journal of Biomedicine and Biotechnology. 2011;2011:1–9.

135. Hutterer A, Betschinger J, Petronczki M, Knoblich JA. Sequential Roles of Cdc42, Par-6, aPKC, and Lgl in the Establishment of Epithelial Polarity during Drosophila Embryogenesis. Developmental Cell. 2004 Jun;6(6):845–54.

136. Tanentzapf G, Tepass U. Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. Nat Cell Biol. 2003 Jan;5(1):46–52.

137. Betschinger J, Mechtler K, Knoblich JA. The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature. 2003 Mar;422(6929):326–30.

138. Chi F, Sharpley MS, Nagaraj R, Roy SS, Banerjee U. Glycolysis-Independent Glucose Metabolism Distinguishes TE from ICM Fate during Mammalian Embryogenesis. Developmental Cell. 2020 Apr;53(1):9-26.e4.

139. Rolls MM, Albertson R, Shih HP, Lee CY, Doe CQ. Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. Journal of Cell Biology. 2003 Dec 8;163(5):1089–98.

140. Martin P. Role of zetaPKC in B-cell signaling and function. The EMBO Journal. 2002 Aug 1;21(15):4049–57.

141. Benirschke K, Kaufmann P. Pathology of the human placenta. 3rd ed. New York: Springer-Verlag; 1995.

142. Boyd JD, Hamilton WJ. The human placenta. Cambridge: Heffer; 1970. 365 p.

143. Renaud SJ, Jeyarajah MJ. How trophoblasts fuse: an in-depth look into placental syncytiotrophoblast formation. Cell Mol Life Sci. 2022 Aug;79(8):433.

144. Tannetta D, Collett G, Vatish M, Redman C, Sargent I. Syncytiotrophoblast extracellular vesicles – Circulating biopsies reflecting placental health. Placenta. 2017 Apr;52:134–8.

145. Walker N, Filis P, Soffientini U, Bellingham M, O'Shaughnessy PJ, Fowler PA. Placental transporter localization and expression in the Human: the importance of species, sex, and gestational age differences†. Biology of Reproduction. 2017 Apr 1;96(4):733–42.

146. Alarcon VB. Cell Polarity Regulator PARD6B Is Essential for Trophectoderm Formation in the Preimplantation Mouse Embryo1. Biology of Reproduction. 2010 Sep 1;83(3):347–58.

147. Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ. Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. J Biol Chem. 1993 Nov 15;268(32):24296–302.

148. Harris TJC, Peifer M. aPKC controls microtubule organization to balance adherens junction symmetry and planar polarity during development. Dev Cell. 2007 May;12(5):727–38.

149. Lee CY, Robinson KJ, Doe CQ. Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature. 2006 Feb 2;439(7076):594–8.

150. Suzuki A, Akimoto K, Ohno S. Protein kinase C lambda/iota (PKClambda/iota): a PKC isotype essential for the development of multicellular organisms. J Biochem. 2003 Jan;133(1):9–16.

151. Puls A, Schmidt S, Grawe F, Stabel S. Interaction of protein kinase C ζ with ZIP, a novel protein kinase C-binding protein. Proc Natl Acad Sci USA. 1997 Jun 10;94(12):6191–6.

152. Joberty G, Petersen C, Gao L, Macara IG. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol. 2000 Aug;2(8):531–9.

153. Zhang H, Neimanis S, Lopez-Garcia LA, Arencibia JM, Amon S, Stroba A, et al. Molecular Mechanism of Regulation of the Atypical Protein Kinase C by N-terminal Domains and an Allosteric Small Compound. Chemistry & Biology. 2014 Jun;21(6):754–65.

154. Fields AP, Gustafson WC. Protein Kinase C in Disease: Cancer. In: Protein Kinase C Protocols [Internet]. New Jersey: Humana Press; 2003 [cited 2022 Sep 9]. p. 519–38. Available from: http://link.springer.com/10.1385/1-59259-397-6:519

155. Wald FA, Forteza R, Diwadkar-Watkins R, Mashukova A, Duncan R, Abreu MT, et al. Aberrant expression of the polarity complex atypical PKC and non-muscle myosin IIA in active and inactive inflammatory bowel disease. Virchows Arch. 2011 Sep;459(3):331–8.

156. Mashukova A, Wald FA, Salas PJ. Tumor necrosis factor alpha and inflammation disrupt the polarity complex in intestinal epithelial cells by a posttranslational mechanism. Mol Cell Biol. 2011 Feb;31(4):756–65.

157. Kravtsov D, Mashukova A, Forteza R, Rodriguez MM, Ameen NA, Salas PJ. Myosin 5b loss of function leads to defects in polarized signaling: implication for microvillus inclusion disease pathogenesis and treatment. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2014 Nov 15;307(10):G992–1001.

158. Nishimura T, Kaibuchi K. Numb Controls Integrin Endocytosis for Directional Cell Migration with aPKC and PAR-3. Developmental Cell. 2007 Jul;13(1):15–28.

159. Yoshihama Y, Sasaki K, Horikoshi Y, Suzuki A, Ohtsuka T, Hakuno F, et al. KIBRA Suppresses Apical Exocytosis through Inhibition of aPKC Kinase Activity in Epithelial Cells. Current Biology. 2011 Apr;21(8):705–11.

160. Hirate Y, Hirahara S, Inoue K, Kiyonari H, Niwa H, Sasaki H. Par- APKC -dependent and -independent mechanisms cooperatively control cell polarity, Hippo signaling, and cell positioning in 16-cell stage mouse embryos. Develop Growth Differ. 2015 Oct;57(8):544–56.

161. Dempsey EW, Luse SA. Regional specializations in the syncytial trophoblast of early human placentas. J Anat. 1971 Apr;108(Pt 3):545–61.

162. Azar C, Valentine M, Trausch-Azar J, Druley T, Nelson DM, Schwartz AL. RNA-Seq identifies genes whose proteins are transformative in the differentiation of cytotrophoblast to syncytiotrophoblast, in human primary villous and BeWo trophoblasts. Sci Rep. 2018 Dec;8(1):5142.

163. Davies R, Griffiths C, Askelund K, Palaiologou E, Cleal JK, Page A, et al. Microvillous tip vesicles may be an origin of placental extracellular vesicles. Placenta. 2022 Jun;123:24–30.

164. Couto A, Mack NA, Favia L, Georgiou M. An apicobasal gradient of Rac activity determines protrusion form and position. Nat Commun. 2017 Aug;8(1):15385.

165. Georgiou M, Marinari E, Burden J, Baum B. Cdc42, Par6, and aPKC Regulate Arp2/3-Mediated Endocytosis to Control Local Adherens Junction Stability. Current Biology. 2008 Nov;18(21):1631–8.

166. Ebnet K, Gerke V. Rho and Rab Family Small GTPases in the Regulation of Membrane Polarity in Epithelial Cells. Front Cell Dev Biol. 2022 Jul 4;10:948013.

167. Etienne-Manneville S, Hall A. Integrin-Mediated Activation of Cdc42 Controls Cell Polarity in Migrating Astrocytes through PKCζ. Cell. 2001 Aug;106(4):489–98.

168. Whyte A. Lectin binding by microvillous membranes and coated-pit regions of human syncytial trophoblast. Histochem J. 1980 Sep;12(5):599–607.

169. Fuenzalida B, Kallol S, Lüthi M, Albrecht C, Leiva A. The polarized localization of lipoprotein receptors and cholesterol transporters in the syncytiotrophoblast of the placenta is reproducible in a monolayer of primary human trophoblasts. Placenta. 2021 Feb;105:50–60.

170. Kingdom J, Huppertz B, Seaward G, Kaufmann P. Development of the placental villous tree and its consequences for fetal growth. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2000 Sep;92(1):35–43.

171. Floridon C. Localization of E-cadherin in villous, extravillous and vascular trophoblasts during intrauterine, ectopic and molar pregnancy. Molecular Human Reproduction. 2000 Oct 1;6(10):943–50.

172. Brown LM, Lacey HA, Baker PN, Crocker IP. E-cadherin in the assessment of aberrant placental cytotrophoblast turnover in pregnancies complicated by pre-eclampsia. Histochem Cell Biol. 2005 Dec;124(6):499–506.

173. Patel K, Nguyen J, Shaha S, Zubkowski A, Riddell M. Loss of cell polarity regulators initiates pyroptosis in trophoblasts at the human maternal fetal interface [Internet]. Developmental Biology; 2022 Jul [cited 2022 Oct 14]. Available from: http://biorxiv.org/lookup/doi/10.1101/2022.06.30.498172

174. Bonilha VL, Finnemann SC, Rodriguez-Boulan E. Ezrin Promotes Morphogenesis of Apical Microvilli and Basal Infoldings in Retinal Pigment Epithelium. Journal of Cell Biology. 1999 Dec 27;147(7):1533–48.

175. Knight M, Redman CWG, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. BJOG:An international journal of O&G. 1998 Jun;105(6):632–40.

176. Matsuzawa K, Akita H, Watanabe T, Kakeno M, Matsui T, Wang S, et al. PAR3-aPKC regulates Tiam1 by modulating suppressive internal interactions. Chernoff J, editor. MBoC. 2016 May;27(9):1511–23.

177. Nishimura T, Yamaguchi T, Kato K, Yoshizawa M, Nabeshima Y ichi, Ohno S, et al. PAR-6–PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1. Nat Cell Biol. 2005 Mar;7(3):270–7.

178. Ramachandran P, Barria R, Ashley J, Budnik V. A critical step for postsynaptic F-actin organization: Regulation of Baz/Par-3 localization by aPKC and PTEN. Devel Neurobio. 2009 Aug;69(9):583–602.

179. Oon CH, Prehoda KE. Asymmetric recruitment and actin-dependent cortical flows drive the neuroblast polarity cycle. eLife. 2019 May 8;8:e45815.

180. Bleicher P, Sciortino A, Bausch AR. The dynamics of actin network turnover is self-organized by a growth-depletion feedback. Sci Rep. 2020 Dec;10(1):6215.

181. Fisher AL, Nemeth E. Iron homeostasis during pregnancy. Am J Clin Nutr. 2017 Dec;106(Supplement 6):1567S-1574S.

182. Scholl TO. Iron status during pregnancy: setting the stage for mother and infant. The American Journal of Clinical Nutrition. 2005 May 1;81(5):1218S-1222S.

183. Huang X, Anderle P, Hostettler L, Baumann MU, Surbek DV, Ontsouka EC, et al. Identification of placental nutrient transporters associated with intrauterine growth restriction and pre-eclampsia. BMC Genomics. 2018 Dec;19(1):173.

184. Huang X, Jain A, Baumann M, Körner M, Surbek D, Bütikofer P, et al. Increased Placental Phospholipid Levels in Pre-Eclamptic Pregnancies. IJMS. 2013 Feb 6;14(2):3487–99.

185. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. Obstetrics & Gynecology. 2020 Jun;135(6):e237–60.

186. Bogard AS, Tavalin SJ. Protein Kinase C (PKC) ζ Pseudosubstrate Inhibitor Peptide Promiscuously Binds PKC Family Isoforms and Disrupts Conventional PKC Targeting and Translocation. Mol Pharmacol. 2015 Oct;88(4):728–35.

187. Martin DDO, Beauchamp E, Berthiaume LG. Post-translational myristoylation: Fat matters in cellular life and death. Biochimie. 2011 Jan;93(1):18–31.

188. Magnuson BA, Raju RVS, Moyana TN, Sharma RK. Increased N-Myristoyltransferase Activity Observed in Rat and Human Colonic Tumors. JNCI Journal of the National Cancer Institute. 1995 Nov 1;87(21):1630–5.

189. Raju RVS, Moyana TN, Sharma RK. N-Myristoyltransferase Overexpression in Human Colorectal Adenocarcinomas. Experimental Cell Research. 1997 Aug;235(1):145–54.

190. Cordeddu V, Di Schiavi E, Pennacchio LA, Ma'ayan A, Sarkozy A, Fodale V, et al. Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. Nat Genet. 2009 Sep;41(9):1022–6.

191. Georgiou M, Marinari E, Burden J, Baum B. Cdc42, Par6, and aPKC Regulate Arp2/3-Mediated Endocytosis to Control Local Adherens Junction Stability. Current Biology. 2008 Nov;18(21):1631–8.

192. Guilbert LJ, Winkler-Lowen B, Sherburne R, Rote NS, Li H, Morrish DW. Preparation and Functional Characterization of Villous Cytotrophoblasts Free of Syncytial Fragments. Placenta. 2002 Feb;23(2–3):175–83.

193. Mu÷sch A, Cohen D, Yeaman C, Nelson WJ, Rodriguez-Boulan E, Brennwald PJ. Mammalian Homolog of Drosophila Tumor Suppressor Lethal (2) Giant Larvae Interacts with Basolateral Exocytic Machinery in Madin-Darby Canine Kidney Cells. Malhotra V, editor. MBoC. 2002 Jan;13(1):158–68.

194. Betschinger J, Eisenhaber F, Knoblich JA. Phosphorylation-Induced Autoinhibition Regulates the Cytoskeletal Protein Lethal (2) giant larvae. Current Biology. 2005 Feb;15(3):276–82.

Appendix 1 : Copyright license for Fig 1.2

CCC Marketplace

This is a License Agreement between Khushali Patel ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Order General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Order General Terms and Conditions below.

Order Date	14-Oct-2022	Type of Use	Republish in a thesis/dissertation
Order License ID	1279274-1		
ISBN-13	9780443063855	Publisher	CHURCHILL LIVINGSTONE
		Portion	Image/photo/illustration

LICENSED CONTENT

Publication Title	Haines & Taylor obstetrical and gynaecological pathology	Country	United Kingdom of Great Britain and Northern Ireland
Author/Editor	Haines, Magnus., Fox, H., Wells, M.	Rightsholder	Elsevier Science & Technology Journals
Date	01/01/2003	Publication Type	Book
Language	English		

REQUEST DETAILS

Portion Type	Image/photo/illustration	Distribution	Worldwide
Number of Images / Photos / Illustrations	1	Translation	Original language of publication
Format (select all that apply)	Print, Electronic	Copies for the Disabled?	No
		Minor Editing Privileges?	No
Who Will Republish the Content?	Academic institution	Incidental Promotional Use?	No
Duration of Use	Life of current edition	Currency	CAD
Lifetime Unit Quantity	Up to 999		
Rights Requested	Main product, any product related to main product, and other compilations/derivative products		

NEW WORK DETAILS

Title	Role of aPKC isoforms in maintaining ST polarity	Institution Name	University of Alberta
		Expected Presentation Date	2022-12-05
Instructor Name	Dr. Meghan Riddell		

ADDITIONAL DETAILS

Order Reference Number	N/A

Appendix 2 : Copyright license for Fig 1.3

Marketplace

This is a License Agreement between Khushali Patel ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Order General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Order General Terms and Conditions below.

Order Date	13-Oct-2022	Type of Use	Republish in a thesis/dissertation
Order License ID	1278866-1	Publisher	BENTHAM SCIENCE PUBLISHERS LTD.
ISSN	15701611	Portion	Image/photo/illustration

LICENSED CONTENT

Publication Title	CURRENT VASCULAR PHARMACOLOGY	Country	Netherlands
		Rightsholder	EUREKA SCIENCE (FZC)
Date	01/01/2003	Publication Type	Journal
Language	English		

REQUEST DETAILS

Portion Type	Image/photo/illustration	Distribution	Worldwide
Number of Images / Photos / Illustrations	1	Translation	Original language of publication
Format (select all that apply)	Print, Electronic	Copies for the Disabled?	No
		Minor Editing Privileges?	No
Who Will Republish the Content?	Academic institution	Incidental Promotional Use?	No
Duration of Use	Current edition and up to 10 years	Currency	CAD
Lifetime Unit Quantity	Up to 999		
Rights Requested	Main product, any product related to main product, and other compilations/derivative products		

NEW WORK DETAILS

Title	Role of aPKC isoforms in maintaining ST polarity	Institution Name	University of Alberta
		Expected Presentation Date	2022-12-05
Instructor Name	Dr. Meghan Riddell		

ADDITIONAL DETAILS

Order Reference Number	N/A