The Thesis Committee for Narmada Lavu Certifies that this is the approved version of the following thesis:

Functional role of GSK3β in p38MAPK mediated human amnion membrane senescence

Committee:

Ramkumar Menon, Ph.D., Mentor, Committee chair

Richard Pyles, Ph.D.

Pomila Singh, Ph.D.

Dean, Graduate School

Functional role of GSK3β in p38MAPK mediated human amnion membrane senescence

by

Narmada Lavu, MD.

Thesis

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

The University of Texas Medical Branch

July, 2019

Dedication

This work is dedicated to my family without whom this would not have been possible…

To my parents who always had faith in me even when I seem to have none;

To my sister who puts up with my shenanigans no matter how old I grow;

To my grandparents who taught me to be bold and to always think outside the box;

Last but not the least, my husband, whose warmth and affection gives me strength to pursue my dreams.

Acknowledgments

I would like to thank my mentor, Dr. Ramkumar Menon, for his constant support and encouragement over the past two years. He has provided me with all the resources and guidance I needed to develop my skills as a researcher. He has encouraged me to develop a scientific mind that will help me in my future endeavors as a physician and scientist. A huge thank you to my 'mini-mentors'- Dr. Samantha Sheller-Miller and Dr. Lauren Richardson. Their science and friendship made my graduate school experience so much more rewarding. I would also like to thank the wonderful laboratory technicians of the OBGyn department (UTMB), especially Talar Kechichian, who always had my back.

I would like to thank my committee members for all their support. Many thanks to the Zelda Zinn Casper scholarship for funding my stipend for the past year at UTMB. Lastly, I would like to thank my family and friends without whose support all of this wouldn't have been possible.

Functional role of GSK3β in p38MAPK mediated human amnion membrane senescence

Publication No._____

Narmada Lavu, M.D.

The University of Texas Medical Branch, 2019

Supervisor: Ramkumar Menon

Oxidative stress (OS) induced stress signaler p38 mitogen-activated protein kinase (p38MAPK) activation and fetal membrane senescence are associated with parturition. Our study determined changes in multipotent pro-cell cycle regulator glycogen synthase kinase (GSK) 3β and its regulation by p38MAPK in effecting senescence to further delineate the molecular mechanism involved in senescence. We report that OS resulted in phosphorylation of GSK3β (inactivation) and p38MAPK (activation) that was associated with cell cycle arrest and senescence in amnion cells. Inhibitors to GSK3β and p38MAPK verified their roles. GSK3β inactivation was associated with nuclear translocation of antioxidant Nuclear factor erythroid 2–related factor 2 (Nrf2) and exosomal section of β-catenin. OS-induced P-p38MAPK activation is associated with functional down regulation of GSK3β and arrest of cell cycle progression and senescence of amnion cells. Lack of nuclear translocation of β-catenin and its excretion via exosomes further supports the postulation that GSK3β down regulation by p38MAPK may stop cell proliferation preceding cell senescence. A better understanding of molecular mechanisms of senescence will help develop therapeutic strategies to prevent preterm birth.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AECs	amnion epithelial cells
AKT(PKB)	protein kinase B
AMCs	amnion mesenchymal cells
AQP	aquaporin
ARE	antioxidant response element
ASK1	apoptosis signal regulating kinase 1
bZip	basic region leucine zipper
COX-2	cyclo-oxygenase-2
CRH	corticotropin-releasing hormone
CSE	cigarette smoke extract
C12FDG	5-dodecanoylaminofluorescein di-β-D-galactopyranoside
ECM	extra cellular matrix
eIF2β	eukaryotic initiation factor 2 beta
ERK	extracellular signal-regulated kinase
EV	extracellular vesicle
GSK3α/β	glycogen synthase kinase 3 alpha or beta
HSP90	heat shock protein 90
HUVEC	human umbilical vein endothelial cells
IF	immunofluorescence
IHC	immunohistochemistry
IL	interleukin
IUGR	intrauterine growth restriction
Keap1	kelch like ECH associated protein 1
MMP	matrix metalloproteinase
mTOR	mechanistic target of rapamycin
MVBs	multivesicular bodies
NF-κB	nuclear factor kappa B
Nrf2	nuclear factor erythroid 2–related factor 2
OS	oxidative stress
PG	prostaglandin
PI3K	phosphoinositide 3 kinase
PPAR	peroxisome proliferator activated receptor gamma
pPROM	preterm premature rupture of membranes
PTB	preterm birth
p38MAPK	p38 mitogen activated protein kinase
ROS	reactive oxygen species
SASP	senescence associated secretory phenotype
SA–β-Gal	senescence associated β Gal
TAB1	TGFβ activated kinase 1 binding protein 1
TGFβ	transforming growth factor β
TL	term-labor
TNF-α	tumor necrosis factor α

TNIL	Term-not-in-labor
WB	western blotting
Wnt	Wingless/Integrated pathway

INTRODUCTION

Chapter 1. Background and significance

EPIDEMIOLOGY AND CAUSES OF PRETERM LABOR

The time taken by the fetus to complete development *in utero* is approximately 40 weeks and is called term gestation. At the end of the gestational period, labor and delivery (parturition) of the fetus, and expulsion of the placenta and fetal membranes occurs. Birth of the fetus before 37 weeks of gestation is called preterm birth (PTB) [1]. Annually, 15 million babies are born preterm around the world, affecting about 5-18% of pregnancies [2]. Despite the medical advancements in the country, 1 out of 10 babies in the United States are born preterm [3]. PTB is one of the leading causes of death amongst neonates. Premature babies that survive have an increased risk of not only short term complications such as respiratory distress [4], but also long term complications including neurodevelopmental disorders and defects in vision and hearing [5].

Preterm labor can be iatrogenic (induced), in cases of maternal-fetal complications during pregnancy, or can be spontaneous, with no known causes, which is characterized by uterine contractions with or without early rupture of fetal membranes. Infection within the amniotic cavity, prior history, behavioral factors, oxidative stress (OS) inducing conditions (poor nutrition, BMI, improper placentation), decidual hemorrhage, race/genetic, and environmental pollutants are some of the risk factors that can potentially activate early contractions of the uterus and cause subsequent delivery [4, 6-8]. However, the signaling pathways and molecular mechanisms underlying preterm labor are still unknown. It is possible that mechanisms that underlie preterm labor may be similar to what happens at term. Thus, understanding the mechanisms of parturition at term should give some insight

into preterm birth. Our project is designed to understand signaling pathways that maybe leading to both term and preterm labor.

PHYSIOLOGICAL MECHANISMS ASSOCIATED WITH HUMAN PARTURITION

Human parturition is a complex, multifactorial process that is regulated tightly by immune, endocrine and neural factors and mechanisms [9]. Some of the accepted theories of human parturition include the following:

1) *'Functional progesterone withdrawal'*: Progesterone is a pro-gestational hormone. Progesterone's suppressive effect on estrogen receptors causes the myometrium to become refractory to circulating estrogen (a pro-labor hormone) [10], and maintains pregnancy. At term, however, correlative changes in the progesterone receptors (increase in progesterone receptor A compared to progesterone receptor B) causes a 'functional' withdrawal of progesterone, causing myometrial contraction and labor [11].

2) *Increase in Prostaglandin levels*: An increase in the bioavailability of prostaglandins (PGE_2, $PGF_2\alpha$) before initiation of labor has been reported [12]. The increase in prostaglandins possibly contributes to fetal membrane and cervical remodeling leading to contraction of the myometrium and labor [13].

3) *Increase in oxytocin and vasopressin*: Uterine contractions are brought about by the increased production of pituitary hormones-oxytocin and vasopressin, by both, the mother and the fetus [14]. Corresponding receptors of vasopressin and oxytocin in the myometrium also increase prior to the onset of labor [15].

4) *Increase in levels of corticotropin releasing hormone (CRH)*: Higher levels of CRH in the amniotic fluid, as well as in maternal serum, towards term gestation contributes to increased fetal cortisol production which promotes fetal lung maturity. CRH also increases the levels of prostaglandin and estrogen in the fetal membranes, decidua and myometrium ultimately leading to labor [9].

5) *Contribution of inflammation*: Higher concentrations of cytokines (including IL-1β, IL-6, and IL-8), tumor necrosis factor α (TNF-α) and transforming growth factor β1 (TGF-β1) in the amniotic fluid, cervical-vaginal fluid, fetal membranes and decidua during term and preterm labor suggests an increase in inflammation [9, 16] compared to respective control samples. Pro-inflammatory cytokines regulate release of prostaglandins, which can initiate labor [17]. In a feed-back loop, cytokines can activate nuclear factor kappa B (NF-κB), which up-regulates the expression of cyclo-oxygenase-2 (COX-2), resulting in an increase in prostaglandins and loss of functional progesterone [18].

Multiple pathways (Fig. 1.1), as discussed above, are proposed to initiate labor. The exact mechanisms and pathways leading to parturition, however, are still elusive. Obtaining human fetal tissues during gestation, for investigative purposes, is difficult and impractical. While one can learn from animal models, animal studies have their own drawbacks, due to variations in endocrine systems governing gestation in different species. Therefore, there is a relative lack of understanding of labor pathways in humans.

Human fetal membranes rupture prior to delivery of the fetus and can be obtained for study following labor. Our laboratory has been examining human fetal membranes from full term or pre-term births to define better mechanisms leading to labor. We have an approved IRB protocol for obtaining fetal membranes from women, who have consented to investigative use of the tissues, following vaginal/cesarean delivery at UTMB. Our laboratory is studying signaling pathways in fetal membranes, as opposed to maternal tissues, in order to obtain novel insights into cellular mechanisms of labor.

MICROARCHITECTURE OF THE FETAL MEMBRANES

Fetal membranes (also called amniochorion or placental membranes), are extra-embryonic tissues surrounding the fetus during gestation [19]. Fetal membranes have endocrine, immune and mechanical functions within the intrauterine cavity [20]. Broadly,

the fetal membranes are divided into morphologically distinct layers, the amnion membrane and the chorion membrane [20, 21].

The amnion membrane is the innermost layer of the fetal membranes towards the fetus [20]. The amnion membrane is avascular and receives oxygen and nutrients from the amniotic fluid that bathes it [22]. The amnion is divided into 5 layers as can be seen in Fig. 1.2 [22].

(1) A single layer of cuboidal epithelial cells forms the innermost layer of the amnion towards the fetus [20]. (2) The basement membrane is formed of collagen types III and IV and non-collagenous glycoproteins secreted by the amnion epithelial cells (AECs). (3) The 'compact layer' is made of collagen types I, III and V secreted by the amnion mesenchymal cells (AMCs) [21, 22]. The interstitial collagens in the compact layer help maintain tensile strength and mechanical integrity of the amnion [22]. (4) The 'fibroblast layer' is the thickest of all the layers of amnion [22] and is composed of AMCs and macrophages embedded in extra cellular matrix (ECM) [22]. The mesenchymal cells are mainly involved in the production and redistribution of ECM needed for structural and cellular support of the membranes during gestation [20]. (5) The 'spongy layer' is the intermediate layer that separates the amnion and chorion [22]. The spongy layer is made up of glycoproteins, proteoglycans and type III collagen and counteracts mechanical stress [21, 22].

The thicker chorion membrane is made up of two layers and is present towards the maternal side [20].

(1) The outer layer, called the trophoblast layer, is composed of the cytotrophoblast and the syncitiotrophoblast [20]. (2) The inner layer of the chorion is made of the somatic mesoderm [20]. Types IV and V collagens predominate in the chorion [21].

Thus, the amniochorion has a distinct and well-defined microarchitecture that helps protect the fetus during gestation. However, fetal membranes have been shown to act not

only as a physical barrier during pregnancy but also to regulate labor and delivery, as discussed below.

FETAL MEMBRANE SENESCENCE AS A MECHANISM FOR INITIATING LABOR.

Our laboratory has demonstrated a novel mechanism of fetal membrane senescence leading to parturition [23-26]. 'Senescence' is the arrest of cell growth that occurs naturally due to aging of a living cell or organism; senescence is irreversible [24]. Fetal membranes can be treated with cigarette smoke extract (CSE), an OS inducer, to mimic term labor. We have reported that treatment of fetal membranes with CSE causes the generation of reactive oxygen species (ROS) that can cause p38 mitogen activated protein kinase (p38MAPK) activation [23, 24]. p38MAPK has multiple functions in the cell, including activation of senescence or aging [25]. Additionally, senescence associated secretory phenotype (SASP), characterized by inflammation with an increase in pro-inflammatory chemokines, cytokines, matrix metalloproteinases (MMPs) and growth factors, was observed in fetal membranes by our laboratory [23]. Inflammatory overload can potentially function as a signal from the fetus, which can cause myometrial contraction and cervical ripening, and initiate labor [23]. In this way, fetal membranes can contribute to labor mechanisms (Fig. 1.3).

REGULATION AND TARGETS OF GSK3B

GSK3 is a serine / threonine kinase and has two structurally similar isoforms, α, and β. The activity of GSK3α/β is regulated by a number of phosphorylation sites [27, 28]. GSK3 is essential for maintaining cellular homeostasis in almost all biological systems [29]. As GSK3β has been noted to be a regulator of multiple pathways in reproductive tissues [29], we specifically studied the β isoform of GSK3. Unlike other kinases, GSK3β is constitutively active and is inactivated upon phosphorylation at the Ser9 site and has maximal activity upon phosphorylation at Tyr216 site [30].

GSK3β's function can be regulated by a number of upstream regulators including Wnt, phosphoinositide 3-kinase (PI3K) / protein kinase B (AKT), and p38MAPK [31-33]. Many of the indicated molecules are part of signaling pathways known to be active during gestation and labor [25, 34-37]. Over a 100 proteins have been identified as possible substrates of GSK3β [38]. β-catenin is an important downstream target of GSK3β. GSK3β phosphorylates and binds β-catenin along with adenomatous polyposis coli, Axin-1, and casein kinase-1, to form the "β-catenin destruction complex" [39]. In the absence of Wnt signaling, β-catenin is phosphorylated by GSK3β, causing ubiquitin-mediated degradation of β-catenin [39]. Phosphorylation of GSK3β at Ser9 phosphorylation site by upstream regulators causes inactivation of the kinase. GSK3β is then unable to phosphorylate β-catenin which becomes free to move into the nucleus and function as a potent transcription factor for up-regulating many pathways associated with, but not limited to, cell survival, proliferation and embryonic development [39, 40].

Another possible downstream target of GSK3β is Nrf2 [38]. Nrf2 is a basic region leucine zipper (bZip) transcription factor which is a major regulator of the antioxidant response of a cell [41]. Nrf2 knockout mice were noted to be highly susceptible to OS induced damage [42]. Canonically, Nrf2 is regulated by Kelch-like ECH-associated protein 1 (Keap1) which is able to sequester Nrf2 in the cytoplasm and cause it's ubiquitination and degradation [42, 43]. OS and inducers of Nrf2 like sulforaphane can cause a conformational change in the structure of Keap1 and prevent the ubiquitination of Nrf2 [44]. Nrf2 can then translocate into the nucleus and bind to the antioxidant response element (ARE) of the DNA to induce a variety of genes, such as heme-oxygenease1, to activate the antioxidant response in the cell [44-46]. A non-canonical or Keap1-independent regulation of Nrf2 includes the ubiquitination and degradation of Nrf2 by GSK3β [47]. The inactivation of GSK3β leading to the activation of Nrf2 has been demonstrated in several studies [47-50]. The involvement of Nrf2 in the OS induced

antioxidant response and senescence in the amnion membrane has been explored in our present study.

CONTRIBUTION OF EXOSOMES TO PARTURITION.

EVs are vesicles made of a lipid bilayer that can carry lipids, proteins, and nucleic acids [51] and can act as a mode of intercellular communication [52]. EVs are believed to be targeted to specific cell types, and signal via receptor ligand interactions or by endocytosis. EVs can also release their contents into the recipient cell after fusing with plasma membrane of the cells [51]. Exosomes are a type of EV that are generated within multivesicular bodies (MVBs) and are secreted out of the cell when MVBs fuse with the cell membrane [51]. Exosomes are less than 150nm in diameter [51]. Since exosomes are small, they can be effectively transported to different cellular layers and act as a means of communication between cells [52]. The content of exosomes reflects the physiologic state of the cells they are derived from [52]. Tumor cells produce a large number of exosomes compared to normal cells and have been studied extensively in cancer biology [53].

Exosomes were originally believed to act as 'garbage bags' [54]. However, now we know that exosomes take part in a number of biological processes including T-cell stimulation, regeneration of adipose tissue, neurotransmission, to name a few [54]. In reproductive tissues, placental exosomes have been characterized, and their functional roles have been studied [55]. Our laboratory was amongst the first to report the contents of fetal membrane derived exosomes [52]. P-p38MAPK (Thr180/Tyr182) was noted in exosomes, and was higher in exosomes derived from AECs treated with CSE (Fig. 1.4) [52]. Our findings suggested that the CSE-treated amnion cells are in a state of senescence, possibly due to OS. Recently, our laboratory demonstrated that exosomes derived from CSE-treated AECs increased inflammation in maternal tissues, including decidua and myometrium [56]. Thus, exosomes carrying specific cargo may have functional relevance in labor.

THE RELEVANCE OF THIS STUDY

A number of theories have been proposed to explain the onset of term and preterm labor [9]. However, the specific mechanisms leading to labor are still elusive. Identifying the signaling molecule(s) (maternal or fetal), which may serve as master regulators of labor initiation, will help develop therapeutic interventions to prevent PTB.

Our laboratory has reported a novel mechanism of premature senescence of the amnion membrane that contributes to preterm labor [7, 57]. We have reported that an increase in OS can cause senescence and inflammation of the amnion membrane, contributing to both term and preterm labor [57, 58]. Mechanistically, OS-induced senescence is mediated through telomere reduction, activation of stress signaler p38MAPK, and development of sterile inflammation [23, 24, 59]. The exact mediators of p38MAPK-induced senescence are uncertain. Therefore, identifying novel upstream and downstream targets of this pathway is critical in elucidating potential preterm birth therapeutics.

In pursuit of these targets, we identified that AECs treated with CSE activated p38MAPK leading to the phosphorylation and inactivation of GSK3β. Targets effected by GSK3β include anti-oxidant nuclear factor erythroid 2–related factor 2 (Nrf2) and pro-cell growth factor β-catenin, reporter of Wnt signaling [28, 52]. However, the specific role of GSK3β in the amnion membrane, in response to p38MAPK activation by OS, remains unknown [29]. We hypothesize that OS in amnion membrane cells activates p38MAPK, leading to inactivation of GSK3β that subsequently causes Nrf2 and β-catenin dependent senescence. Additionally, secreted products from the amnion cells, like exosomes, can impact senescence outcome. This hypothesis was tested in the following specific aim:

Specific Aim: To determine the role of p38MAPK-GSK3β signaling in OS mediated senescence in AECs and AMCs. Primary AECs and AMCs, isolated from term caesarean fetal membranes, were exposed to CSE. Phosphorylation of p38MAPK and

GSK3β, and downstream targets of GSK3β (β-catenin and Nrf2), were examined by western blotting (WB). Effect of OS on cell cycle and senescence was analyzed by flow cytometry. Pharmacologic inhibitors of p38MAPK and GSK3β provided experimental rigor. This aim helped determine the p38MAPK-GSK3β networking, its impact on downstream pathways, and AEC and AMC cell fate in response to OS. The experiments in this study also helped examine a possible contribution of exosomes to the senescence of amnion cells.

Although various interventions have been tried to prevent preterm labor, the outcomes are not always clinically effective. Understanding the functional role of important regulators of biological processes will help to identify if one or more molecules (p38MAPK, GSK3β, etc.) can serve as therapeutic target(s) for preventing PTB. Thus, our studies are expected to address some of the gaps in our knowledge in this field, with important translational value.

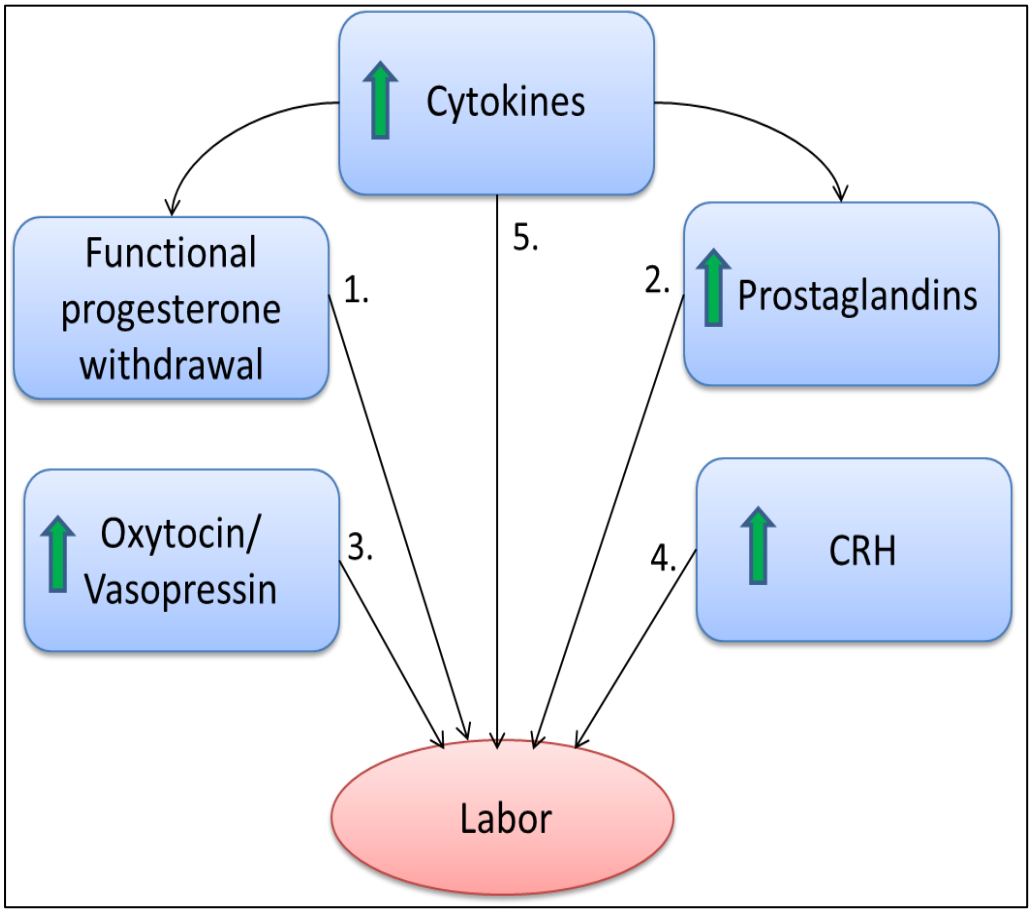


Fig. 1.1 Schematic diagram of possible mechanisms leading to labor.

Of the number of theories that have been proposed to explain the possible mechanism leading to labor some of the most accepted ones include: 1. a functional withdrawal of progesterone hormone, 2. an increase in prostaglandins, 3. an increase in Oxytocin hormone, 4. an increase in CRH or 5. an increase in cytokines at the end of the gestational period (adapted from Kamel et al, Arch Gynecol Obstet, 2010).

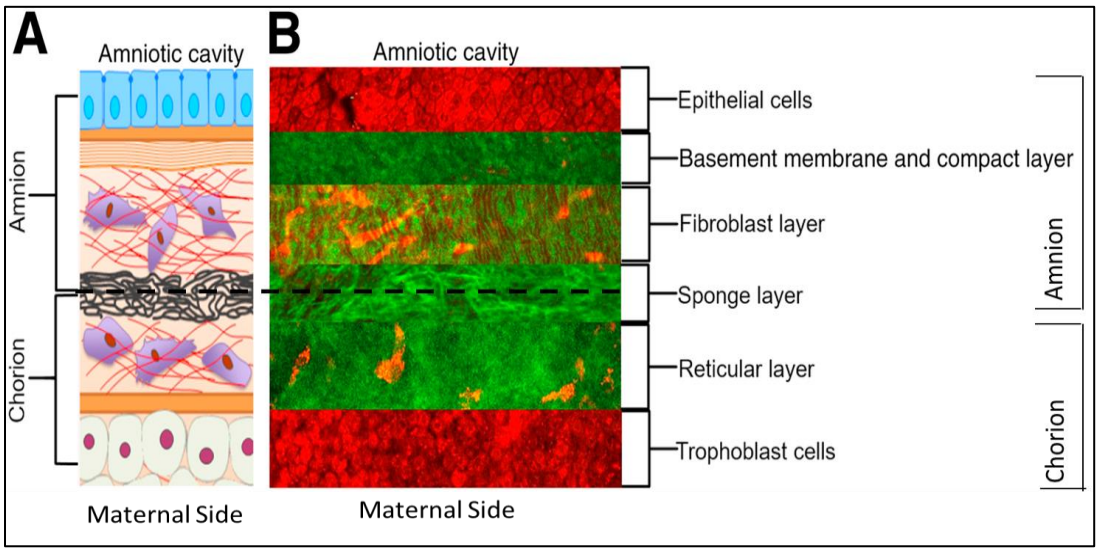


Fig. 1.2 Fetal membrane microarchitecture.

A. Diagrammatic representation of the cellular layers of the amniochorionic membranes. B. Fetal membrane layers as visualized by multiphoton autofluorescence microscopy and second harmonic generation microscopy (L. Richardson et al, Am J Pathol, 2017).

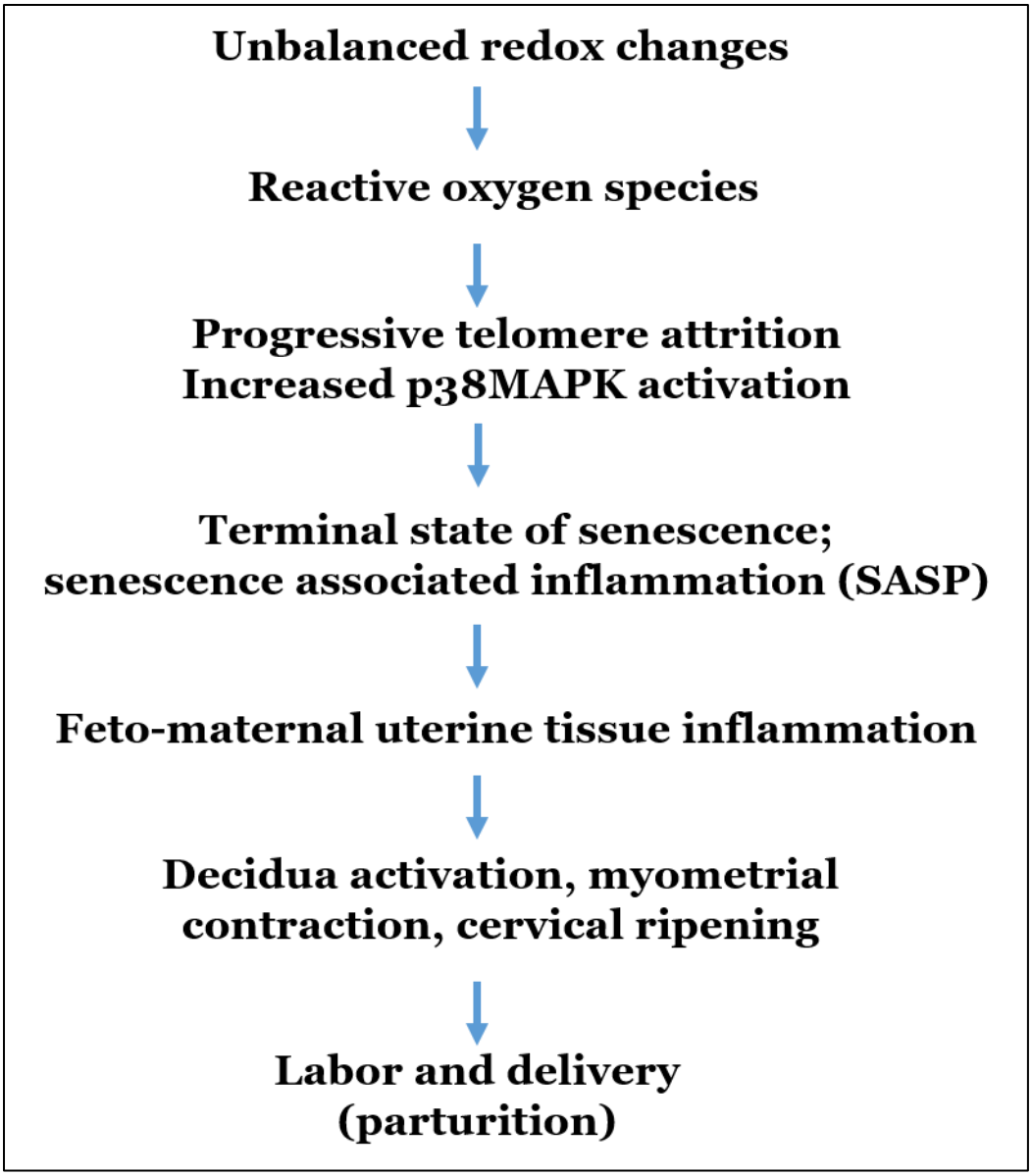


Fig. 1.3 Flowchart depicting the role of p38MAPK in fetal membrane senescence and labor.

An increase in the levels of ROS within the intrauterine cavity has been demonstrated to cause telomere attrition and p38MAPK activation that can contribute to fetal membrane senescence and sterile inflammation (senescence associated secretory phenotype; SASP). Inflammation can then be propagated to the maternal tissues to ultimately cause delivery of the fetus (adapted from R. Menon et al, Hum Reprod Update, 2016).

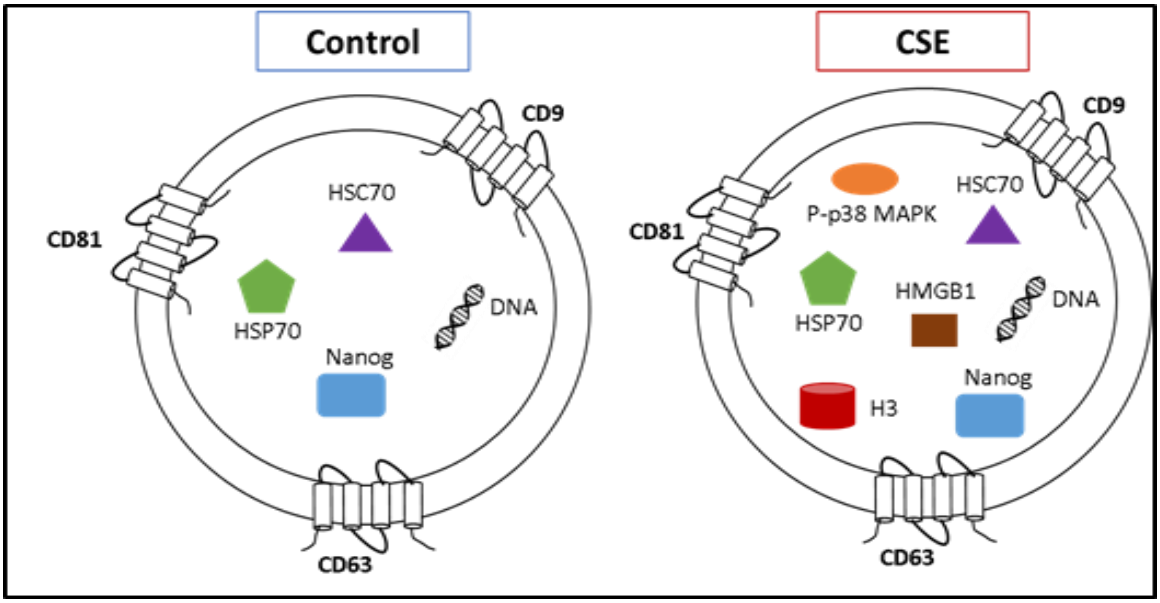


Fig. 1.4 Molecules (cargo) present within exosomes from control and CSE-treated AECs.

The cargo within exosomes isolated from AECs is representative of the physiologic state of the cell of origin. Exosomes derived from CSE-treated AECs demonstrated an increased amount of activated p38MAPK compared to exosomes derived from control AECs (S. Sheller et al., PLoS One, 2016).

Does GSK3β have a functional role during pregnancy and parturition?

GSK3β was originally identified as a kinase important during biosynthesis of glycogen [60]. Now we know that the role of GSK3β cannot be restricted to metabolism alone. GSK3β has caught the attention of the scientific community in the recent past as it has been demonstrated to play an integral role in the biological processes of almost all organs in the body [61]. Thus, dysfunction of the kinase is known to contribute to a multitude of pathologies- including neurodegenerative disease, psychiatric illnesses, cardio vascular disorders, diabetes and cancer [30, 60, 62].

Since GSK3β's role in human disease has been widely accepted, its role as a potential therapeutic target is being increasingly explored. Despite the discovery of multiple phosphorylation sites and possible substrates for GSK3β, the mechanisms of regulation by different upstream regulators as well the specificity of these regulators to the different phosphorylation sites of GSK3β are still elusive. Thus a number of studies are being undertaken to better understand the structure, regulation and possible substrates of this multifunctional kinase [38, 61].

Having such a wide range of applications in other fields of study and organ systems, GSK3β is bound to play an important role in reproductive biology in general and pregnancy and parturition in particular. In order to understand the extent of functional and mechanistic studies involving GSK3β during pregnancy and parturition, we conducted a systematic review of literature. Following the review, we understood that the number of studies on GSK3β during pregnancy and labor are few and far between. The systematic review helped us understand several knowledge gaps in the field **(chapter 2)** which helped design our subsequent experiments as we explored the role of GSK3β in OS induced fetal membrane senescence **(chapter 3)**.

Chapter 2. Glycogen synthase kinase (GSK) 3 in Pregnancy and Parturition: a Systematic Review of Literature [29]

INTRODUCTION

Human reproduction is a complex process reliant on multitudes of cellular signaling molecules for proper implantation, development, and delivery of a healthy fetus. Understanding these regulatory molecules, their expression, and their function in various reproductive tissues is important to determine their contributions to various pregnancy-related functions. Aberrant expressions and functions of these molecules can contribute to an abnormal pregnancy environment and adverse outcomes [63-65]. GSK3 is one such key cell function regulatory molecule. GSK3 is a serine / threonine kinase, which is regulated by multiple phosphorylation sites (Fig. 2.1) and functions in a number of processes in the cell [28]. GSK3 exists as 2 isoforms, α (alpha) and β (beta), which have structural similarities within their kinase domain [27] and molecular weights of 51 kd and 47 kd, respectively [66]. Subtle differences arise in N- and C-terminal sequences [27], suggesting distinct biological functions [67].

There are many upstream regulators of GSK3's function in response to a variety of signals. Some of the main GSK3 regulators include Wnt, PI3K / AKT, extracellular signal-regulated kinase (ERK), and p38MAPK. These pathways are reported to play important roles in pregnancy maintenance as well as term and preterm births [25, 34-37]. Close to 100 proteins have been suggested to serve as GSK3's substrates [38]. GSK3 can be found in the cytosol, mitochondria, and nucleus [30] or bound to complexes, such as the "β-catenin destruction complex," [68] a molecule widely reported to promote cell survival. The "β-catenin destruction complex" includes GSK3, adenomatous polyposis coli, Axin-1, casein kinase-1, and cytosolic β-catenin [39]. In the absence of Wnt signaling, β-catenin

gets phosphorylated by GSK-3, causing ubiquitin-mediated degradation of β-catenin that threatens cell survival [39].

Like GSK3, p38MAPK is a multifunctional kinase that controls various cellular functions, including cell growth and development and stress-associated cell death. Recent reports from our laboratory [7, 26], and many others, have noted that stress responder p38MAPK plays a major role in regulating cellular functions in response to ROS during parturition. ROS-mediated and p38MAPK-induced senescence (mechanism of aging) is a salient feature of parturition at term and preterm. p38MAPK also has the ability to functionally control other primary and secondary signaling molecules, like GSK3 via phosphorylation, which suggests that, at term, active p38MAPK could phosphorylate [[69, 70]; Fig. 2.1] and suppress GSK3. This inactivation can lead to an accumulation of β-catenin and the promotion of cell survival transcription factors. An increase in β-catenin could play a homeostatic role between p38MAPK-induced senescence and GSK3-regulated cell survival, allowing intrauterine tissues to survive till delivery. However, due to the large number of target substrates for GSK3 and regulators that control GSK3's function, the exact functional role of GSK3 is hard to determine.

A systematic review of literature was conducted to determine a comprehensive functional role of GSK3 in reproductive tissues and to identify knowledge gaps to derive a better hypothesis in future experiments. Our objectives were to 1) identify reports on GSK3-associated functional changes in human and animal pregnancy and parturition, 2) determine the mechanistic roles of GSK3 reported in human and animal pregnancy and parturition, and 3) determine the knowledge gaps, if any, in GSK3's functional role in human and animal pregnancy and parturition.

METHODS

A systematic review of the literature was conducted as per the requirements of the MOOSE group and PRISMA statement [71, 72]. This systematic review was registered in PROSPERO (Registration number: CRD42018081241).

Search criteria for identification of studies

A systematic review of the literature published in English from 1980–2017 was collected from 3 databases, Ovid, Web of Science, and clinicaltrials.gov, with the assistance of a librarian team at The University of Texas Medical Branch at Galveston.

Search strategy

A search strategy was developed to study GSK3's expression, function, and mechanistic role in the intrauterine compartments and other reproductive organs of interest (ovaries, implanted blastocysts, fetal membranes, placentas, and uteruses) during gestation (implantation through development) and parturition (at term or preterm delivery).

Selection criteria

Types of studies: This review was restricted to studies primarily focusing on GSK3 in humans and all animal models of pregnancy. We selected original research studies that investigated GSK3 in various intrauterine components during implantation, pregnancy, and parturition. Studies were excluded if they were not related to GSK3; not related to the reproductive period, specifically embryogenesis and contraception; review articles; or below average quality score; they were also excluded if the full text was not available (either published as an abstract only or not obtained from authors upon request). Studies were included if they reported samples from subjects at full-term gestation (≥ 37 weeks) who were either in labor or not in labor at the time of collection. Studies were also included if results were solely related to patients with preterm labor and delivery (both spontaneous and induced) or chorioamnionitis or if they utilized cell lines from gestational tissues

irrespective of maternal age, fetal gender, sociodemographic and other clinical factors, geographic location, and ethnicity and race.

Types of outcome measures: Two types of reported outcome measurements were extracted for data analysis: 1) expression and functional changes associated with GSK3 in reproductive tissues and 2) mechanistic roles of GSK3 during implantation, pregnancy, and parturition in human and animal models. Specifically, we examined studies that assessed GSK3's mRNA or protein expression changes, its activators and repressors, its mechanisms of activation or inactivation, and biological pathways impacted in response to changes in GSK3's function.

Data collection and analysis

Selection of studies: All citations retrieved through the search were downloaded to a common storage folder where 2 authors (N.L. and L.R.) independently screened the titles and abstracts. Titles and abstracts that were not related to GSK3, articles that did not fit our inclusion criteria (ie, reproductive tissues in implantation, pregnancy, and parturition), and review articles were removed, and duplicates were excluded. The studies that fulfilled the selection criteria were included for full-text review and data extraction.

Data extraction: A data extraction form was created to collect the following information: study's primary author, year of publication, journal of publication, country of origin for the primary author, assessment of GSK3's isoforms, assay type used to determine GSK3's expression or function, gestational tissue studied, and the findings and/or end phenotype measured in response GSK3's activity. Two authors (N.L. and L.R.) independently extracted data from the included reports, compared their findings, resolved disagreements through discussion, and produced a single final form for each included study.

Quality assessment: Quality assessment tools for systematic reviews have not been well defined for basic science research. Therefore, guidelines recently described by Hadley

et al [73] were used. Criteria used for quality improvement included the following: hypothesis-driven study designs and approaches; strength of study design as defined by subject ascertainment methods; description of sample collection, processing, and storage; description of reagents used for specific assays with detailed protocol that are sufficient to reproduce data; scientific rigor with details on appropriate controls and statistical tests; and sufficient explanation of results and whether the results supported the conclusions (Supplemental Fig. 2.1). There criteria were analyzed in each article, and a score was given to each one, ranking it as poor, acceptable, or good quality.

Data synthesis

Based on our inclusion / exclusion criteria, data that reported GSK3's role in tissues of interest (implanted blastocysts, fetal membranes, placentas, ovaries, and uteruses) during implantation, pregnancy, and parturition, either in humans or animal models, were gathered. Based on these, we synthesized the functions of GSK3 in each one of these tissues and documented the major upstream regulators, downstream targets and functions, and project knowledge gaps yet to be filled.

RESULTS

Our search yielded 738 citations. After screening citations by title, 149 articles remained, which were again screened by abstract. A total of 80 studies were included for a full-text review. After removing duplicates, 59 studies remained. After the full-text review, 25 studies were included for the final data extraction and analysis (Table 2.1; Fig. 2.2).

For our chosen timeframe, the first GSK3 article was published in 2005 and the largest number of GSK3 publications were found in 2013 (Fig. 2.3A). As expected, the reports evaluated were 99% basic science or research laboratory-based studies, with only 1 paper related to a clinical case [74]. In the latter report, high serum alkaline phosphatase

in a 37-week pregnant patient revealed an increased rate of cytotrophoblast proliferation due to inactivation of GSK3β downstream to AKT activation.

Quality Assessment

Twenty-one articles (84%) were graded as good quality, and the rest (4; 16%) were graded as acceptable quality; none were graded as poor (Fig. 2.3B-C). Good quality articles provided adequate descriptions of sample ascertainment, study objectives, materials and methods, assay and analytical strategies, and data.

Main characteristics of studies

The main characteristics and findings of the included studies are summarized in Table 2.1. GSK3 studies were conducted by researchers from many different institutions around the globe, but predominantly from Europe (40%) and North America (32%). Reports included in our review investigated biological processes involved in pregnancy (starting from implantation) and parturition and tissues and cells from both fetal and maternal compartments.

Methods used for detecting GSK3's expression and function

The mechanistic roles of GSK3 that were analyzed included determination of activators or pathways causing its activation. Functional changes of GSK3 were investigated as either pathways or the endpoint resulting from GSK3's activation. Most studies determined total GSK3 and its isoforms' (α and/or β) expressions by WB analysis. Total GSK3 expressions were reported through qPCR [74, 75], and GSK3's activation was determined using indirect immunoperoxidase analysis [76]. Phosphorylated forms of GSK3, specifically Ser21 for GSK3α and Ser 9 and Tyr 216 for GSK3β, were studied using WB analysis, immunohistochemistry [77], indirect immunoperoxidase analysis [76], and also by bio-plex phosphoprotein detection kit [63]. To confirm the functional roles of

GSK3 and to provide scientific rigor to their experimental approaches, many studies included inhibitors of GSK3, like CHIR99021 [78], LiCl [77], and siRNA [65] (Table 1).

Main findings

Studies included in our final review reported multiple reproductive tissues derived from a number of different species. Among all the reports, 18 studies used tissues / cells from humans, 2 used mice, 2 used sheep, 1 used rat, 1 used bovine, and 1 used equine. We also found that 12 reports conducted studies using placental cells or tissues, 8 used fetal, 6 used maternal uterine and 2 used ovarian (Table 2.1).

Most GSK3 studies in reproductive tissues were conducted in placental tissues and cells, indicating a role of GSK3 in trophoblast invasion and differentiation [74, 79-85]. GSK3's role in the pathogenesis of certain disorders during pregnancy, especially preeclampsia and intrauterine growth restriction (IUGR), were also reported [64, 77, 86-88]. Studies of GSK3's role in other tissues included the following: ovaries for establishment of pregnancy [34, 89], endometrium for decidualization [76, 90], and the fetal membranes and myometrium for initiation of labor [78]. Interestingly, there were no reports of GSK3 in the cervix or the vagina.

GSK3 was reported to be involved in numerous signaling pathways in these reports. Thus, there was more than 1 upstream regulator and downstream target for GSK3. The most common upstream regulator was AKT. AKT was most commonly activated by PI3K [63, 79, 82-84, 88], but could also be activated by other less common activators, like Galectin 1 [91], hepatocyte growth factor [87], or was part of the AKT-mechanistic target of rapamycin (mTOR) pathway [86, 92] (Table 2.1). GSK3 was also shown to be a part of the Wnt- or ROS-signaling pathways [93, 94]. GSK3 has multiple downstream targets that can lead to a number of different outcomes in reproductive tissues and cells. Eight studies reported β-catenin as a downstream [34, 63, 64, 76, 80, 83, 85, 87] target of GSK3 promoting cell survival, while eukaryotic initiation factor 2 β (eIF2β) [84, 86] was the

second most common downstream target documented in 2 studies (Table 2.1), suggesting GSK3 mediates protein synthesis and cell proliferation.

Our review noted that GSK3 was studied as a part of a major signaling pathway either downstream or upstream to another molecule of interest and not necessarily as the primary molecule of interest for investigation. In placental tissues or cells, GSK3 was reported to be a part of the AKT pathway where it aids in trophoblast differentiation [74, 79, 81], trophoblast invasion [82-84], and likely in the pathogenesis of preeclampsia [64, 77, 86, 87] and IUGR [86, 88]. GSK3 as a part of the Wnt-signaling pathway was also shown to play a role in trophoblastic invasion using placental cells [85]. Trophoblast migration was studied in endometrial tissues and conceptus trophectoderm, where GSK3 was reported to aid AKT pathway by promoting translation via eIF2β [84]. In ovarian tissues, the AKT-GSK3 pathway was involved in pregnancy establishment [34].

As mentioned above, only a handful of studies examined GSK3 as their primary molecule of interest and investigated its functional role. Using fetal membranes as a model, Lim et al recently reported that GSK3 may play a major role in the terminal processes of human labor at term and preterm, where GSK3's increased activity leads to an increase in pro-labor and pro-inflammatory cytokines [78].

Astuti et al showed that recombinant relaxin was able to induce proliferation and cell survival in HTR-8/SVneo cells (transformed extravillous trophoblast) via PI3K-AKT activation and GSK3β phosphorylation, leading to cell survival [79]. Roseweir et al reported that a complex signaling pathway involving GSK3β might act as a negative regulator of trophoblast cell migration to possibly restrict the amount of invasion at the time of placentation [80].

Rider et al demonstrated that decidualization may be controlled by an endocrine-dependent Wnt signaling, where progesterone downregulates GSK3β via the Wnt pathway in uterine tissues [76]. Also, in bovine luteal cells, Hou et al have shown that luteinizing

hormone via phosphorylation of GSK3β amongst other mechanisms regulates progesterone synthesis, which is critical for the maintenance of pregnancy [89].

Hua et al reported that Tanshinone IIA, extracted from the root of *Salvia miltiorrhiza*, stimulates aquaporin (AQP) expression (especially AQP8) in amnion Wish cells via phosphorylation of GSK3β and may be a possible mode of treatment for oligohydramnios [65]. Feng et al noted that human AECs increase mitochondrial permeability in response to an exposure to a 50-Hz magnetic field [93], an effect mediated by ROS/GSK3β-signaling pathway [93]. This frequency is important because it is emitted from most cell phones and computers. Other studies that reported about glucose transport in endometrial tissues [95] and placentas [75] and possible causes of gestational diabetes mellitus in human umbilical vein endothelial cells (HUVEC) [94], placental tissue preservation [92], have also been included in the review.

Summarily, our review found that most studies included an incidental investigation of GSK3's function in many reproductive systems. The number of studies that look exclusively at GSK3's mechanistic and functional role in reproductive tissues are few and far between.

DISCUSSION

Twenty-five studies reporting GSK3's role during pregnancy and parturition were analyzed for this review. Human and animal origin studies used tissues/cells from the uterus and ovary as well as those from fetal origins (placentas, fetal membranes, and HUVEC). In these reports, AKT-, Wnt-, and ROS-signaling pathways were reported predominantly as regulators of GSK3's expression and function, while β-catenin was the most commonly reported downstream target (Table 2.1).

Based on our systematic review, we report that GSK3 is a major intermediary regulator of signaling pathways in a number of biological processes involved in pregnancy

and parturition, including establishment of pregnancy and blastocyst implantation, trophoblast migration and invasion, decidualization, placental glucose transport, and complications of pregnancy, such as gestational diabetes mellitus, IUGR, preeclampsia, oligohydramnios, and labor initiation at term and preterm. Each of these processes may be activated by different agents / stimulants via separate signaling pathways that involve GSK3. Understanding and pinpointing GSK3's exact function in a particular tissue is quite challenging due to various reasons: 1) the existence of multitudes of regulators, which phosphorylate and, in the process, modify the functional activity of GSK3, 2) the large number of substrate targets, and 3) multiple downstream effecters. Even though important biological processes during pregnancy and parturition have been studied, GSK3's role in other important events, like placental growth and its regulation under various oxidative conditions, remodeling of fetal membranes during pregnancy, cervical ripening, myometrial activation, etc., have not been reported.

Although GSK3 exists in 2 isoforms, α and β[27], our review found that most reports were based on β isoform only, although the choice of β isoform for their studies was not properly rationalized. Only 6 of the studies reported and commented on both the isoforms [63, 64, 78, 81, 88, 92]. It might be possible that GSK3β is the most studied form due a literature bias, suggesting that mammalian GSK3β was more effective than GSK3α [96, 97]. Availability of reagents (primarily antibodies) to study GSK3β may also have influenced this choice. These reports, however, did not equalize the level of expression of the 2 isoforms, hiding the fact that α and β are redundant in terms of regulating Wnt/β-catenin signaling [27].

GSK3 is part of a number of signaling pathways in reproductive tissues (Fig. 2.4). The upstream regulators may phosphorylate GSK3 at multiple sites—most commonly the Serine 21 for the α isoform and serine 9 and Tyr 216 for the β isoform. Only 2 studies looked at the Tyr 216 phosphorylation [77, 94], while 3 studies did not report specific phosphorylation sites examined in their experiments [34, 74, 87]. Also, from studies

conducted in other tissues, GSK3 was phosphorylated at the T390 residue in humans as part of the p38MAPK pathway [70]. However, though p38MAPK was found to play a role in trophoblast migration [79], membrane senescence and parturition, IUGR development [88], and PE [74], none of the studies included in this review examined this pathway or this particular phosphorylation site.

ROS-induced p38MAPK is a signaling pathway noted to be active in reproductive tissues at term [7, 26]. However, none of the studies in the review linked GSK3 and p38MAPK as a regulator of GSK3 function. Two of the studies showed that ROS [93, 94] can be an upstream regulator of GSK3. However, no connection between the 2 has been made in any of the studies that we reviewed. This might be an important link, especially in determining the labor mechanism at term and preterm. This is a major knowledge gap to be investigated in future research. Following this review, we concluded that GSK3 is an important regulator that plays a major role in a variety of biological and metabolic processes in reproductive tissues (Fig. 2.4). One of the goals of this systematic review is to project lack of research in this area and to encourage investigators to build their research agenda around this pluripotent functional molecule. Targeting this molecule for therapeutic purposes in some of the obstetric complications discussed might prove to be fruitful. However, further studies need to be conducted to understand how GSK3's regulation may affect cellular processes in reproductive cells, especially when GSK3 acts as a major intermediary between a number of signaling pathways, with possible crosstalk between these pathways. Also, researchers need to be extremely wary of specific upstream regulators, phosphorylation sites, and downstream targets that ultimately define a particular action of GSK3 prior to initiation of studies.

CONCLUSION

GSK3's role in various utero placental tissues has been studied in connection with pregnancy and parturition. GSK3 studies in reproductive biology are predominantly confirming its well-reported activators or downstream effectors. Novel concepts are hard to derive to define the functional role of GSK3 due to multiple reasons; 1. Very few studies have been conducted in GSK3 to elucidate its mechanisms of action in reproductive tissues 2. Many reports are not replicated by multiple laboratories or details are lacking to replicate reported data. 3. GSK3 has multiple phosphorylation sites and based on a specific site, functional changes are expected. Clarification of these sites is generally lacking and lack of details makes it difficult to derive accurate conclusions. Function of GSK3 is dependent on the site-specific phosphorylation / modifications (activation / inhibition). Pregnancy and parturition involve several unique changes, specifically constant changes in oxidative radicals and well-regulated localized inflammation that are required to maintain tissue growth and integrity. GSK3's role in maintaining tissue homeostasis during microenvironmental changes has hardly been studied. The association between GSK3's altered function contributing to pathological pregnancy outcomes has not been reported. Current reports are not convincing enough to define a precise role due to a lack of scientific rigor in experimental approaches. Mechanistic and functional studies are essential to better understand the contributions of a critical cell function regulator. Understanding the role of GSK3 throughout gestation and in normal term pregnancies can provide insight into pathologic activation of its pathways that can cause adverse pregnancies.

FUTURE DIRECTIONS

This systematic review has shown us that GSK3 is now emerging as a major regulator of biological functions in reproductive tissues. However, studies that look primarily at GSK3 as the molecule of interest are few. Further studies that will delineate

the mechanistic role of GSK3 in reproductive tissues are needed to better understand if GSK3 might serve as a potential target for therapeutic interventions in adverse pregnancy events.

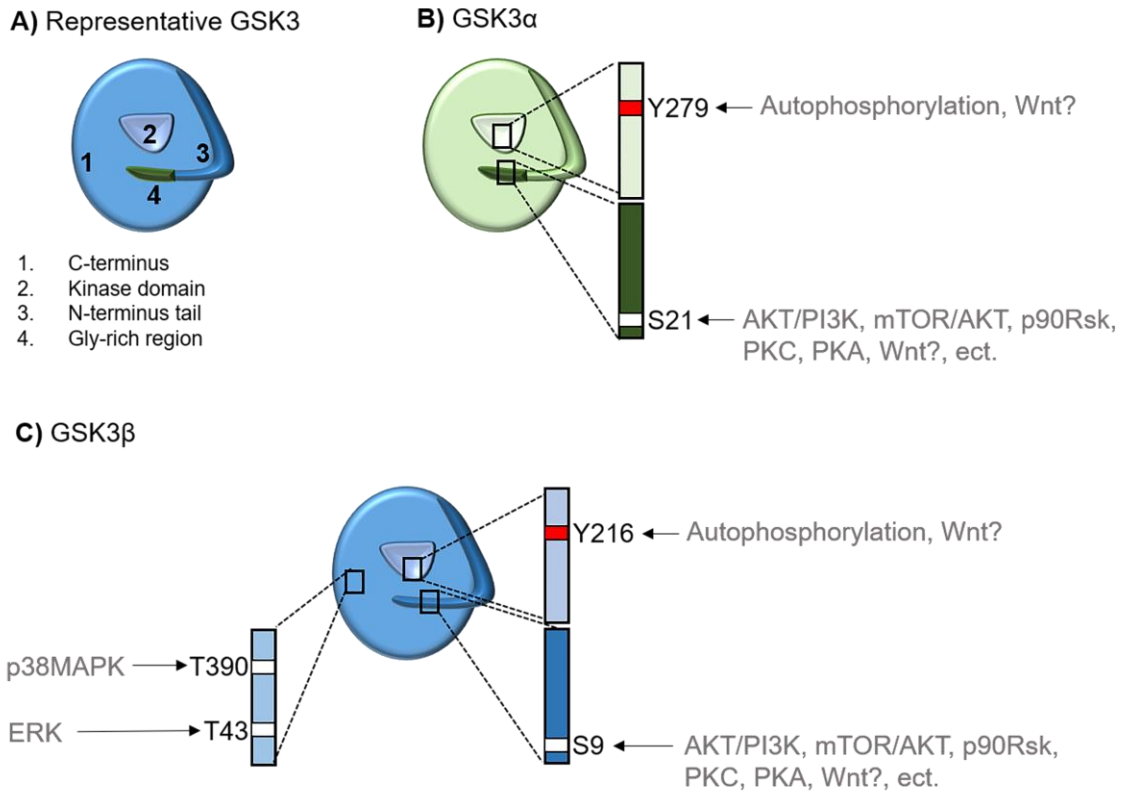


Fig. 2.1 Graphical representation of GSK3 isoform phosphorylation sites.

A) Common regulatory sites of GSK3α/β. B) Representative structure of GSK3α containing a inhibition phosphorylation site (white) S21 on its N-terminal tail which can be phosphorylated by AKT/PI3K, mTOR/AKT, p90Rsk, PKC, PKA, and potentially the Wnt pathway. The N-terminal tail can act as a pre-phosphorylated substrate, or pseudosubstrate when stimulated by these upstream kinases. GSK3α also contains an over activation site in its kinase domain, Y279, which has been documented to be active by autophosphorylation. C) Representative structure of GSK3β containing a inhibition phosphorylation site (white) S9 on its N-terminal tail which can be phosphorylated by AKT/PI3K, mTOR/AKT, p90Rsk, PKC, PKA, and potentially the Wnt pathway. GSK3β also contains an over activation site in its kinase domain, Y216, which has been documented to be active by autophosphorylation. Uniquely, p38MAPK (T390) and ERK (T43) can also inhibit GSK3β by phosphorylating it on its C-terminus.

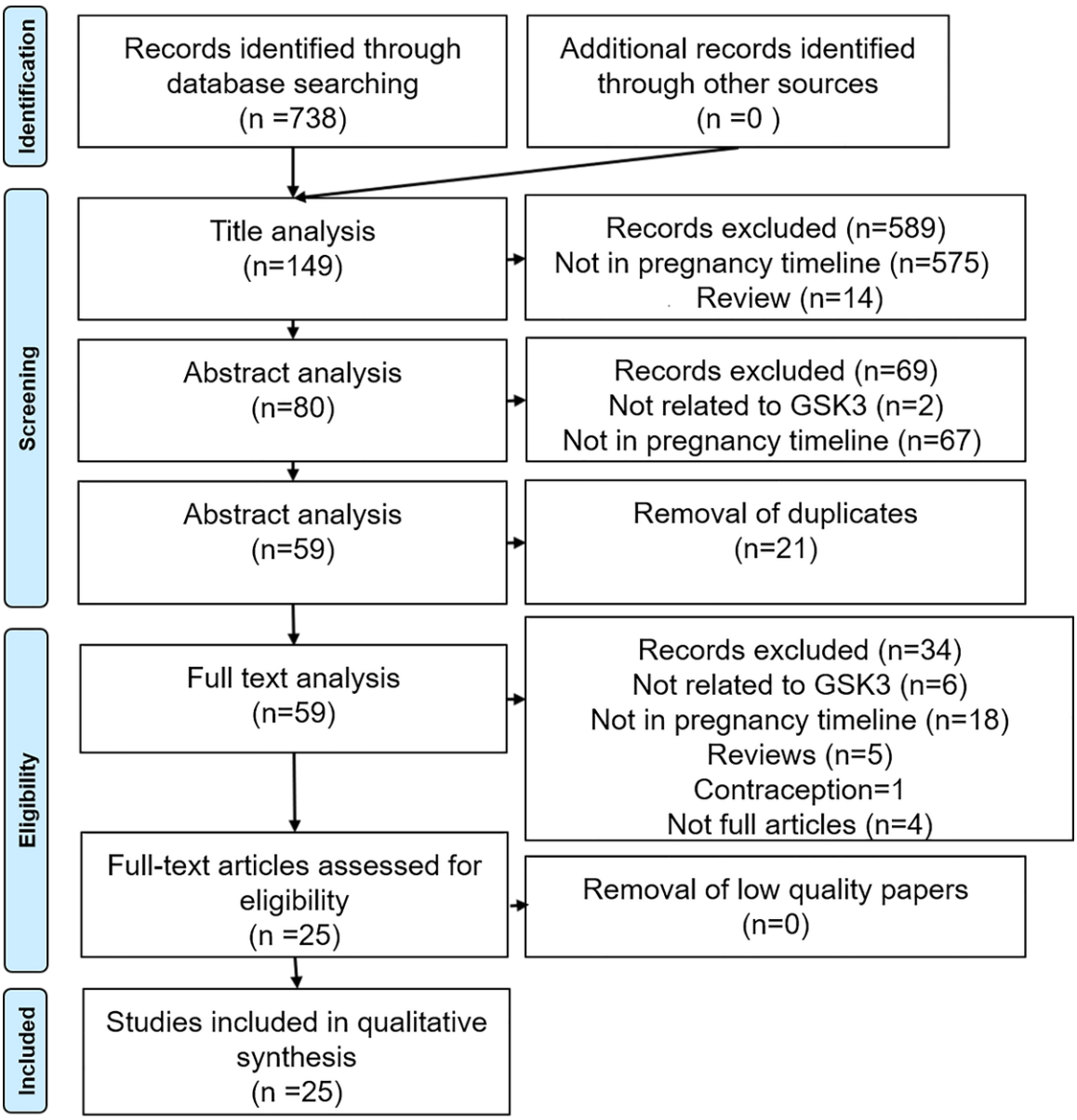


Fig. 2.2 PRISMA flow chart

PRISMA flow chart documenting GSK3 systematic review search strategy.

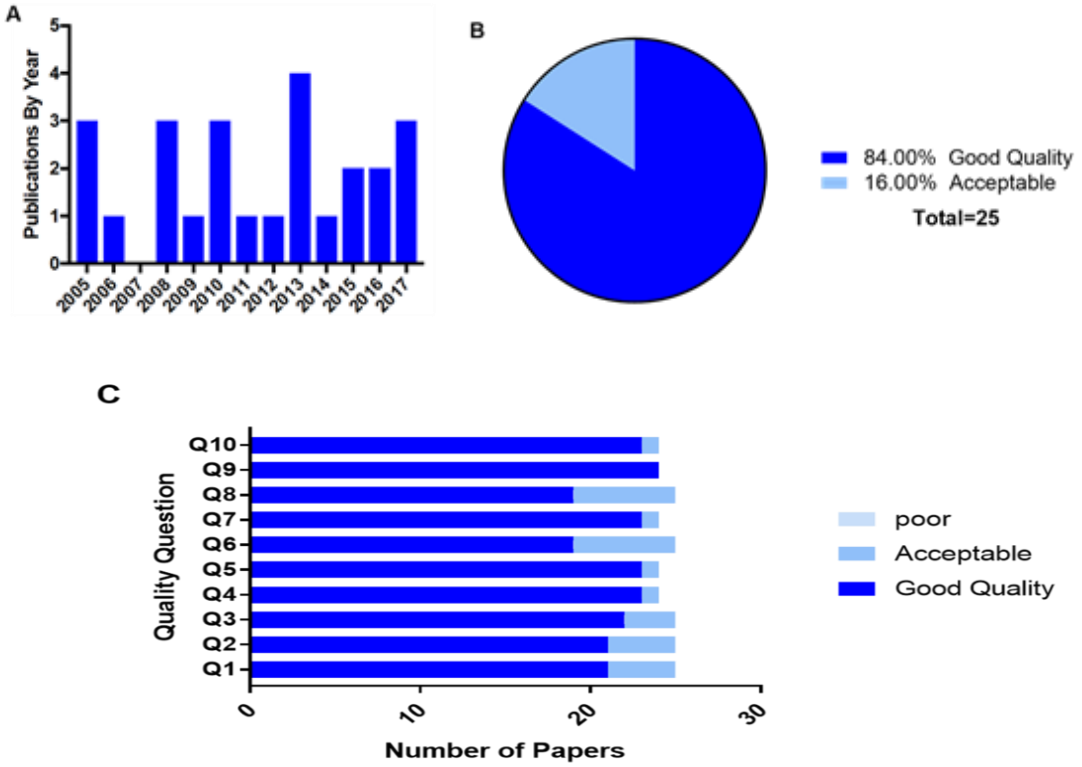


Fig. 2.3 Systematic review quality assessment

Quantity of GSK3 papers per year meeting our criteria. B) Percent of final articles from our systematic review that scored ‘good quality’ or ‘acceptable’. No papers were scored poor quality. C) Scores of each of our final 25 papers broken down per question.

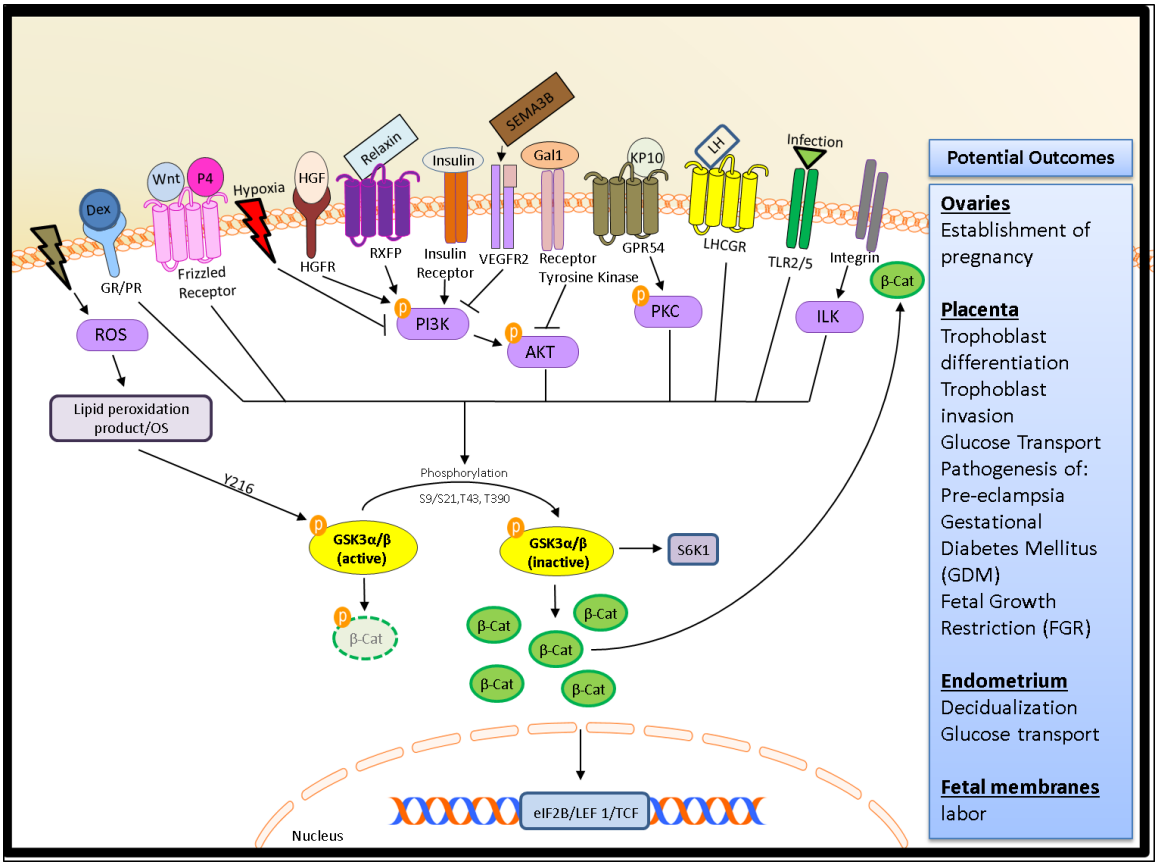


Fig. 2.4 Proposed mechanistic pathways of GSK3 (Glycogen Synthase Kinase 3) and reported functional outcomes.

Hormones, infections, signaling molecules and other environmental triggers like oxidative stress are all possible stimulants to the GSK3 pathways. They interact with the cells either directly or by binding to their specific receptors, and are able to cause phosphorylation of GSK3 through mediators like PI3K/AKT, PKC and ILK. Phosphorylation at S9 causes inactivation of GSK3 while phosphorylation at Y216 keeps it in its active state. Phosphorylation may also occur at other sites (including T390 and T43) based on the upstream regulators. Inactivation of GSK3 can lead to accumulation of β-catenin which can then either translocate into the nucleus and cause activation of specific transcription factors or attach to integrin and bring about changes in the ECM. Multiple targets of GSK3 have been identified which can ultimately bring about functional outcomes in reproductive tissues including establishment of pregnancy, labor and pathogenesis of diseases like GDM and preeclampsia.

OS: Oxidative Stress, ROS : Reactive Oxygen Species, GR/PR : Glucocorticoid Receptor/Progesterone Receptor, Dex: Dexamethasone, Wnt: Wingless/Integrated ,P4: Progesterone, HGF: Hepatocyte Growth Factor, HGFR: Hepatocyte Growth Factor receptor, RXFP: Relaxin Family Peptide Receptors, SEMA3B: Semaphorin 3B, VEGFR2: Vascular endothelial Growth Factor receptor 2, Gal1:Galectin-1, KP10:Kisspeptin 10, GPR54: G-protein Coupled Receptor 54, LH: Luteinizing Hormone, LHCGR: Luteinizing Hormone/ Choriogonadotropin Receptor, TLR: Toll-like receptor, ILK: Integrin linked Kinase, β-Cat: β-catenin, PKC: Protein Kinase C, AKT: Protein Kinase B, PI3K:

Phosphoinositide 3-kinase,S6K1: Ribosomal protein S6 kinase beta-1,eIF2B: eukaryotic initiation factor 2, LEF1: Lymphoid Enhancer Binding Factor 1,TCF: Transcription Factor, IL6/8: Interleukin 6/8, MMP-2: Matrix Metalloproteinase-2.

Authors	Country	Year	Species studied	Type of Study	Type of Sample	Methods	Upstream GSK3ß	Form of GSK3	Down Stream Function
Lee et al.	United States	2016	Ewes	Establishment of pregancy	Oaries were collected and the CL isolated	WB	EGR-->AKT	P-GSK3β No phosphorilation site mentioned	β-Catenin and P-CREB, leads to programed survial and establishment of pregancy
Astuti et al.	Japan	2015	Human	Trophoblast differentiation	HTR-8/Svnevo and human extravillous trophoblast	WB	PI3K and AKT	P-GSK3β S9	Surivival
Yung et al.	U.K	2014	Human	Tissue preservation (Other)	Placenta	WB	AKT-mTOR	P-GSK3α/β S21/S9	
O'Connell et al.	Australia	2013	Mice	Glucose transport	Placenta	PCR	Dexamethosone	Just total GSK3β	GYS1 and GBE1
Cheng et al.	U.K	2013	Human	Gestational diabetes mellitus (GDM)	Endothelial cells-HUVEC (Human umbilical vein endothelial cells)	WB	ROS	P-GSK3β Y216	Defects in NrF2 necular accumlation and its down stream targets NQO1, Bach1, GCLM, and xCT
Roseweir et al.	Uk	2012	Human	Trophoblast differentiation and invation	First trimester extravillious trophoblasts (HTR8SVneo)	WB	Kisspeptin-10	P-GSK3β S9	β-Catenin in cytoplasum and enhance the effects on migration
Lague et al.	Canada	2010	Mice	Trophoblast invasion	Decidua	WB	PI3K/AKT	P-GSK3β S9	Represed decidua cells and limited transformation
Yung et al.	U.K	2008	Human	PE and IUGR	IUGR and PE placenta and JEG-3 cells	WB	AKT-->mTOR	P-GSK3β S9	eIF2Bε, 4E-BP1, and Eef2k
Rider et al.	United States	2006	Rats(Ovariectomized)	Decidualization in early pregnancy	Uterine tissue (uterine horns from OVX rats)	Indirect immunoperoxidase analysis and WB	Wnt	P-GSK3β S9	β-Catenin and TCF/LEF
Boronkai et al.	Hungary	2005	Human	Syncitiotrophoblastic differentation	Placental tissue, umbilical cord, and membranes	WB	Phospho-AKT	P-GSK3β No phosphorilation site mentioned	
Bramer et al.	Canada	2017	Mares (Horse)	Glucose transport	Endometrial tissue	PCR	Just total GSK3β		
Kim et al.	United States	2008	Sheep	Trophoblast migration	Endometrial tissue,conceptus trophectoderm	WB	PI3K/AKT	P-GSK3β S9	eIF2B

Table 2.1 Systematic Review Results

Can GSK3β play a role in Oxidative stress response and senescence of fetal membranes?

As evident from the systematic review, in reproductive tissues, GSK3β has been reported to play an important role in various biological processes right from implantation of the blastocyst and development of the fetus and placenta, up to delivery of the baby [29, 80, 98-100]. The role of GSK3β during the process of labor has also been reported by Lim et al. recently [78].

The extensive literature review helped us identify several knowledge gaps as well as challenges associated with the study of GSK3β. Multiple upstream regulators dictate the downstream function of GSK3β, with possible crosstalk between the pathways. Thus, understanding the exact functional and mechanistic role of GSK3β in the tissue of interest is a challenge. Although biological processes that are important during gestation and labor have been studied, the role of GSK3β in critical processes like cervical and fetal membrane remodeling, activation of the myometrium, cervical ripening, etc., has not been examined [29]. OS-induced p38MAPK activation, which leads to fetal membrane senescence and inflammation, has been demonstrated by our laboratory [7, 26]. Results from studies in other fields indicate that p38MAPK acts as an upstream regulator of GSK3β [33, 101]. However, the role of p38MAPK in regulating GSK3β's function in reproductive tissues has not been studied. We have, for the first time, examined the mechanistic and functional role of GSK3β and its contribution to senescence in the amnion membranes as well as it's regulation by p38MAPK as discussed in **chapter 3**.

Chapter 3. Oxidative Stress Induced Downregulation of Glycogen Synthase kinase 3β (GSK3β) in Fetal Membranes Promotes Cellular Senescence [102]

INTRODUCTION

Fetal membranes (amniochorion or placental membranes) are layers of extra-embryonic tissue that function as a protective barrier during pregnancy [19, 103]. Fetal membranes undergo senescence, an irreversible arrest of cell growth that occurs naturally contributing to aging [23, 24]. An increase in OS, at term, or at preterm within the intrauterine compartment has been experimentally shown to cause senescence and inflammation of the amniochorion tissue [25, 26, 59]. OS-induced fetal membrane senescence has been established as a plausible factor associated with labor not only at term but can increase the risk of PTB and preterm premature rupture of membranes (pPROM) as well [24, 58].

OS-induced fetal membrane senescence is mediated through telomere reduction, activation of stress signaler p38MAPK and development of sterile inflammation called senescence-associated secretory phenotype (SASP). OS experienced by fetal membranes can result from a non-infectious (often seen at term prior to labor) or infectious (often seen at preterm) stimulant; however, extent of senescence and senescence-associated inflammation may vary based on the type of endogenous or exogenous stimulant. SASP from senescent fetal membranes are predominantly pro-parturient biochemical mediators that can cause activation of maternal tissues such as the uterus and decidua and initiate labor [23]. Mechanistically, one of the initiators of this process in fetal membranes is OS-induced increased expression and accumulation of transforming growth factor β (TGFβ). TGFβ through TGFβ receptor-TGFβ activated kinase 1 binding protein (TAB) 1 complex triggers a non-canonical pathway leading to activation of p38MAPK by

autophosphorylation [104]. A constitutive and modest level of p38MAPK expression is likely during gestation and this is expected to be a tightly regulated process to avoid premature senescence activation. The mechanism of balanced p38MAPK function during gestation and its increased activity contributing to senescence at term is unclear. This knowledge is critical to understand fetal membrane homeostasis during pregnancy and its imbalance contributing to pathologies associated with adverse pregnancies.

In an attempt to further elucidate mechanisms of senescence in the amnion layer of the fetal membranes, this study examined the role of GSK3β. P38MAPK has been demonstrated to regulate the function of GSK3β in other fields [101, 105]. GSK3β is a serine / threonine kinase that is important for maintaining cellular homeostasis by regulating a number of important biological processes [27, 29, 30]. Unlike other kinases, GSK3β is constitutively active and is inactivated upon phosphorylation at the S9 site and has maximal activity upon phosphorylation at Y216 site [30, 106]. Multiple upstream regulators like the Wnt pathway, PI3K / AKT, and p38MAPK dictate GSK3β's downstream function [32, 99, 105]. Of the multitude of GSK3β's downstream targets, β-catenin is an important one [29, 107]. Phosphorylation and inactivation of GSK3β can cause nuclear translocation of β-catenin which can then up-regulate pathways associated with cell survival and proliferation amongst others [39, 40]. Nrf2, which is a transcription factor that plays a role in the antioxidant response of a cell, is another important downstream target of GSK3β [41, 107, 108]. GSK3β is part of the non-canonical pathway of regulation of Nrf2 where inactivation of GSK3β can lead to activation and nuclear translocation of Nrf2 to cause its downstream effect [47].

In reproductive tissues, GSK3β has been reported to play an important role in various biological processes right from implantation of the blastocyst and development of the fetus and placenta, up to delivery of the baby [29, 80, 98-100]. Lim et al. recently reported that an increase in activity of GSK3 in fetal membranes causes an increase in cytokine expression (IL-6, IL-8, TNF α) leading to labor [78]. However, the possible

contribution of GSK3β to OS mediated p38MAPK mediated senescence of fetal membranes has not been studied.

The present study aimed to understand the role of GSK3β in the OS-associated fetal membrane senescence in general and amnion cell senescence in particular and the labor pathways. The regulation of GSK3β's function by p38MAPK and possible downstream targets of GSK3β including β-catenin and Nrf2 were analyzed. We demonstrate that p38MAPK can lead to the inhibition of GSK3β by S9 phosphorylation. Further, GSK3β inhibition caused senescence of amnion cells- a function that can potentially be mediated by Nrf2. Further mechanistic studies are needed to determine the exact downstream target of GSK3β that mediates senescence in fetal membranes.

MATERIAL AND METHODS

Institutional review board approval: Placentas for this study were collected after term vaginal (Term Labor; TL) and scheduled cesarean deliveries (Term Not In Labor; TNIL) from John Sealy Hospital (University of Texas Medical Branch (UTMB) at Galveston, Texas according to the inclusion and exclusion criteria defined by our laboratory [59, 109]. As discarded placentas were used after delivery for the study, subject recruitment or consenting was not done. The Institutional Review Board at UTMB approved the study protocol and placentas were collected according to the regulations of the IRB as an exempt protocol that allowed utilization of the discarded placentas (UTMB 11-251).

Clinical samples: Fetal membranes (amniochorion) were obtained from TNIL scheduled cesarean deliveries (n=12) and TL vaginal deliveries (n=12) as described previously by our laboratory [109]. Briefly, fetal membranes were dissected from the placenta and any adherent blood clots were removed by washing the membranes in normal saline followed by thorough cleaning using sterile cotton gauze. 6mm biopsies

(explants) were collected from the midzone of the fetal membranes. Intact fetal membrane explants (amniochorion), as well as explants with amnion and chorion layer separated, were collected and processed to perform immunohistochemistry and western blotting.

Isolation and culture of human Amnion Epithelial Cells (AECs) and human Amnion Mesenchymal Cells (AMCs): Human AECs were isolated and cultured as previously described by our laboratory [59, 104, 110]. A total of twelve placentae were collected for preparing primary cells used for this study. Briefly, fetal membranes obtained from term scheduled cesarean deliveries (TNIL) were received by our laboratory and approximately 10g of the amnion layer was separated from the chorion layer. The amnion was rinsed in saline and cut into small pieces of approximately 2 cm x 2cm in size. The amnion was digested using with 0.125%collagenase and 1.2% trypsin (Sigma-Aldrich, St. Louis, MO, USA) in Hanks' balanced salt solution (HBSS; Mediatech Inc., Manassas, VA, USA) for 35 minutes. After the digestion step, the tissue was filtered through a 70-μm cell strainer (Thermo Fisher Scientific, Waltham, MA, USA), and trypsin was inactivated with AEC culture medium. Complete AEC medium contained Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 medium (DMEM/F12; Mediatech Inc.) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 10% penicillin/streptomycin (Mediatech Inc.), and 100 μg/mL epidermal growth factor (EGF; Sigma-Aldrich). The above digestion step was repeated once more and the final filtrate was centrifuged at 3000 rpm for 10 minutes. The pellet was resuspended in complete AEC medium and the AECs were cultured in T75 flasks at approximately 3-5 million cells/flask [104]. The flasks were incubated at 37°C, 5% CO2, and 95% air humidity until they reached 70%-80% confluence and were ready to be passaged and treated.

Human AMCs were isolated from the fetal membranes as per the protocol described previously by Kendal-Wright et al. [111, 112] with slight modifications. Briefly, fetal membranes obtained from term scheduled cesarean deliveries (TNIL) were received by our laboratory and approximately 10g of the amnion layer was separated from the chorion

layer. The blood clots adherent to the amnion were removed by rinsing the membrane 3-4 times in HBSS. Incubation of the amnion with 0.05% trypsin/EDTA (Corning, Corning, NY) for 1 h in a 37 °C water bath helped remove AECs. The membrane was then washed 3-4 times in cold HBSS and incubated for 1 hour in a rotator in a digestion buffer consisting of Minimum Essential Medium (Corning), 1 mg/mL collagenase type IV, and 25 μg/mL DNase I. Following complete digestion of the membrane, the solution was neutralized with complete AMC culture medium. Complete AMC medium consists of DMEM/F12 medium supplemented with 5% FBS, 100 U/ml penicillin G, and 10% penicillin/streptomycin. After filtering through a 70 μm cell strainer, the solution was then centrifuged for 10 minutes at 3000 rpm. The cell pellet was resuspended in complete AMC medium and the AMCs were seeded into T75 flasks at a density of 3-5 million cells per flask. The flasks were incubated at 37°C, 5% CO2, and 95% air humidity until they reached 70%-80% confluence and were ready to be passaged and treated.

Cell culture treatments: Cells were passaged into T25 flasks once they reached 70-80% confluence. All experiments were conducted utilizing AECs and AMCs at passage 1. Approximately 1 million AECs/ T25 or 800,000 AMCs/ T25 were plated for 6-hour treatments and 1 million AECs/ T25 or 800,000 AMCs/ T25 were plated for 48-hour treatments. Prior to each treatment, the cells were incubated in serum-free medium for 1 hour. The following treatments were performed in this study: media (control), oxidative stress inducer cigarette smoke extract (CSE) (6 hours 1:10; 48 hours 1:50), CSE+ functional p38 MAPK inhibitor SB203580 (30 μmol/L; Sigma Aldrich #S8307), GSK3β inhibitor CHIR99021 (5 μM; Sigma Aldrich #SML-1046) and as a positive control lithium chloride (20 mmol; Sigma Aldrich # 62476). GSK3β inhibitor concentration for treatments was determined based on cell viability titration studies conducted for this study as well as previously published reports [113]. The cells were incubated after treatment at 37°C, 5% CO2, and 95% air humidity for 6 hours or 48 hours based on the experiment requirements.

CSE treatment: CSE was utilized to induce OS in AECs and AMCs as previously described by our laboratory [59, 104, 114]. CSE is used as an OS-inducing laboratory reagent and not used to test the effect of cigarette smoke as a risk factor associated with pregnancy complications. To produce CSE, smoke from a single commercial cigarette (unfiltered Camel, R.J. Reynolds Tobacco Co, Winston Salem, NC) was bubbled through 25 mL of AEC or AMC medium. A 0.25mm Steri-flip filter unit (Millipore, Billerica, MA) was used to sterilize the stock CSE. The prepared stock CSE was diluted to 1:10 in complete DMEM/F12 medium and utilized for 6-hour treatments and diluted to 1:50 in complete DMEM/F12 medium and utilized for 48-hour treatments of the cells.

Protein extraction and WB: AECs, AMCs, and explant tissue were lysed with RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, and 1.0 mM EDTA pH 8.0, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail and phenylmethylsulfonyl fluoride as described previously by our laboratory [104]. The nuclear and cytoplasmic extraction reagents (NE-PER; Thermofisher #78835) were used, according to the manufacturer's instructions, to extract the protein in the nuclear and cytoplasmic compartments of the control and CSE treated AECs and AMCs. Determinations of protein concentrations were done using Pierce BCA Protein Assay Kit BCA (Thermo Scientific, Waltham, MA, USA). WB was performed as previously described by our laboratory [59, 104]. Briefly, samples were run on gradient (4%–15%) SDS-PAGE Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) and transferred to the membrane using Biorad Gel Transfer Device (Biorad). Membranes were blocked in 5% nonfat milk in 1x Tris buffered saline-Tween 20 (TBS-T) for a minimum of 1 hour at room temperature before adding primary antibody and incubating overnight at 4°C. Appropriate secondary antibody conjugated with horseradish peroxidase was used to incubate the membranes after primary antibody incubation and immunoreactive proteins were visualized using chemiluminescence reagents ECL western blotting detection system (Amersham Piscataway, NJ, USA). The restore western blot stripping buffer (Thermo

Fischer) protocol was used before re-probing the blots and blots were not used more than three times. The primary antibodies used for WBs were: Total GSK3β (1:1000, Cell Signaling, Danvers, MA), P- GSK3β (S9) (1:1000, Cell Signaling), total β-catenin (1:1000, Cell Signaling), Nrf2 (1:500, Abcam, Cambridge, United Kingdom), P-p38MAPK (T180/Y182) (1:300, Cell Signaling), p38MAPK (1:1000, Cell Signaling), Heat Shock Protein 90 (HSP-90, 1:1000, Thermo Fischer Scientific, Rockford, IL), CD-81 (1:400, Abnova, Taipei City, Taiwan), β-Actin (1:15,000, Sigma-Aldrich, St. Louis, MO). The relative levels of the proteins in the specific bands were normalized densitometrically, using associated β-actin levels in the samples, using the Biorad-Image Lab 6.0 software.

Flow cytometry analysis: *Senescence analysis:* AECs and AMCs were treated and incubated for 48 hours prior to analysis for cell senescence using flow cytometry. Senescence-associated β-Galactosidase (SA–β-Gal), a biomarker for senescence, was detected by the flow cytometric SA–β-Gal assay as described previously by our laboratory [59, 114]. Briefly, cells were incubated for 1 hour each in medium with 100 nM bafilomycin A1 (Baf A1) and 6 μmol/L of 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG, eBioscience/ Thermo Fischer Scientific, Waltham, MA). Selection of viable cells was made possible by utilizing DNA Prep Stain which contains propidium iodide (Beckman Coulter). CytoFlex flow cytometer (Beckman Coulter, Indianapolis, IN) was used for the assay and the corresponding CytExpert software (Beckman Coulter) was used for data analysis.

Cell cycle analysis: To determine the pattern of the cell cycle in control and treated cells, flow cytometric cell cycle analysis was performed as described previously [52] with modifications. The Coulter cell cycle kit (Beckman Coulter) was used for this experiment. Briefly, the cells were collected after treatment and spun at 3000 rpm for 10 minutes. The cell pellet was fixed using 70% ethanol and then centrifuged for 5 minutes at 300g. The cell pellet was resuspended in 250 μL of the cell cycle kit and incubated for 15 minutes at room temperature. The samples were then run on the Cytoflex flow cytometer and the

corresponding Cytexpert software was used for analysis. Cycle analysis by measuring DNA content was used to distinguish between different phases of the cell cycles (sub-G0G1, G0G1, S, and G2).

Immunohistochemistry (IHC): IHC was performed on TL and TNIL fetal membrane samples using methods previously described by our laboratory [109, 115]. Briefly, fetal membrane sections were fixed for 48 hours in 4% paraformaldehyde (PFA) and embedded in paraffin. Tissue sections of 5-μm thickness were cut and adhered to a positively charged slide. Xylene was used to deparaffinize the tissues and 100% alcohol, 95% alcohol, and normal saline (pH 7.4) was used for rehydration before proceeding to staining. Five images for each specimen were taken at 20x and 40x magnification. The following primary antibodies were used for IHC: Total GSK3β (1:800, Cell Signaling, Danvers, MA), P- GSK3β (S9) (1:50, Cell Signaling), Total β-catenin (1:200, Cell Signaling).

Isolation and characterization of exosomes: Exosomes were isolated from the collected culture media according to the protocol established and published by our laboratory [52, 56, 114], with slight modifications. Briefly, collected media was centrifuged in a Sorvall Legend X1R and TX-400 swinging bucket rotor (Thermo Fisher Scientific) (2000g for 20 minutes at 4°C), to remove dead cells and cellular debris. Supernatants from the centrifugations were concentrated in Amicon-Ultra 15 tubes (centrifugation at 4000 x g for 30 min) and then filtered in a Nalgene prefilter plus filter. This was followed by centrifugation for 30 mins at 10,000g and ultracentrifugation in a Beckman Optima LX-80 ultracentrifuge with a 70.1Ti rotor (Beckman Coulter) for 2 hours at 100,000g. The pellet was re-suspended in 1×PBS and subjected to size exclusion chromatography through an Exo-spin column to remove co-precipitants that may contaminate the sample. The exosomes were stored at -80^{0}C. The size and concentration of collected exosomes were determined using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany), with the help of the corresponding software (ZetaView 8.02.28;

Particle Metrix). WB analysis was utilized to determine the exosome marker CD81 (1:400, Abnova, Taipei City, Taiwan).

Immunofluorescence (IF) and confocal microscopy: To localize β-catenin within exosomes, IF was performed for the exosomal marker CD81 and β-catenin. AECs were passaged and seeded into Millicell EZ slides 8-well glass slides (Millipore, Billerica, MA) at a concentration of 70,000 AECs/ well. After 24 hours, the cells were treated with either control medium or medium with CSE (1:50 dilution) for 48 hours. IF staining and colocalization experiments were performed as described earlier by our laboratory [52, 114]. Briefly, 4% PFA was used for fixation and 0.5% Triton X was used for permeabilization of the cells. After blocking for 30 minutes in 3% bovine serum albumin (BSA, Fisher Scientific, Waltham, MA) in PBS, primary antibodies (β-catenin 1:1000 dilution in 3% BSA, Cell Signaling and CD81 1:250 dilution in 3% BSA, Abnova) were added and the slides incubated at 4°C overnight. Slides were incubated in secondary Alexa Fluor 488- or 594-conjugated antibodies (Life Technologies, Carlsbad, CA) for one hour at 1:1000 dilution in 3% BSA for β-catenin and 1:400 dilution in 3% BSA for CD81. NucBlue® Live ReadyProbes® Reagent (Life Technologies) for 3 minutes stained the nucleus for DAPI following washes with PBS. Confocal images (five images per condition) were obtained using a Zeiss LSM-880 confocal microscope with a 63 × 1.20 numerical aperture oil immersion objective. Z-stack acquisition was carried out with 0.41-μm z-steps. Image processing and analysis were performed with Fiji (open source). A linescan was traced over the areas in the membrane and cytoplasm where the highest intensity of the colocalized signal was noted (region of interest). Raw fluorescence intensity vs calibrated distance (micrometers) along the linescan was plotted graphically using image J. Pearson correlation coefficient was obtained for the region of interest using coloc2 from Fiji for control and CSE-treated cells.

Statistical analysis: Statistical analyses for normally distributed data were performed using a Student t-test. Statistical values were calculated using GraphPad Prism

7 software (GraphPad Software, Inc., LaJolla, CA, USA). P values equal to or less than 0.05 were considered statistically significant. Data in graphs are represented as Mean ± SEM.

RESULTS

GSK3β and β-catenin are localized in fetal membranes: Fetal membranes are broadly divided into morphologically distinct layers, the amnion membrane and the chorion membrane each with its own well-defined microarchitecture [21]. In order to understand the localization of GSK3β and one of its major downstream targets, β-catenin, in the different layers of the fetal membranes, IHC was performed. Both P-GSK3β, the inactive form of GSK3β, and total GSK3β were localized in all layers of the fetal membranes in both TNIL as well as TL fetal membranes (Fig. 3.1A, 3.1B, 3.1D, 3.1E). Similarly, β-catenin was also localized to all layers of the fetal membranes- the amnion epithelial and mesenchymal layers as well as the chorion mesenchymal and trophoblast layers in both TNIL and TL fetal membranes (Fig. 3.1C, 3.1F). Prior studies from our laboratory had demonstrated that p38MAPK, an upstream regulator of GSK3β was expressed in all layers of the fetal membranes as well [7] and hence IHC of p38MAPK was not repeated in this study. These data provided evidence that both forms of GSK3βs and β-catenin were constitutively expressed in fetal membranes at term; however, this approach did not indicate their quantitative changes to determine any functional relevance associated with their localization.

TL is associated with increased expression of inactive GSK3β in fetal membranes: In order to understand if the relative expression levels of GSK3β, β-catenin and p38MAPK change in TL compared to TNIL fetal membranes, WB was performed. Confirming our prior data, P-p38MAPK was two-fold higher in TL compared to TNIL fetal membranes (p=0.09) (Fig. 3.1G) [7]. The activation of p38MAPK corresponded to inactivation of

GSK3β where we noted a two-fold increase in P-GSK3β in TL fetal membranes when compared to TNIL fetal membranes and this trended towards significance (p=0.07) (Fig. 3.1H). The relative levels of β-catenin, however, did not change between TL and TNIL fetal membranes (p=0.3) (Fig. 3.1I). This data suggests that the p38MAPK/ GSK3β signaling pathway was possibly activated during the process of labor with no change in the levels of β-catenin.

Localization of P-GSK3β and total GSK3β to all layers of the fetal membranes and their increased expression at TL compared to TNIL membranes, prompted us to determine whether their differential expression was limited specifically to the amnion or chorion layers. WBs were done to assess the differential expression of P-p38MAPK, P-GSK3β and β-catenin in the amnion, chorion and intact fetal membranes. We found that the expressions of P-p38MAPK, P- GSK3β and β-catenin were higher in amnion than chorion and intact fetal membrane in both TL and TNIL tissues (data not shown). The data suggests that the p38MAPK/ GSK3β signaling pathway is possibly more active in cells in the amnion layer of the fetal membranes. Therefore, further characterization of GSK3β mechanistic studies were conducted using amnion membrane cells (AECs and AMCs).

OS-induced by CSE increased p38MAPK activation and GSK3β inactivation in AECs and AMCs: Next, we performed primary cell culture experiments to confirm our tissue findings. AEC and AMC markers and morphological characterization using regular microscopy confirmed the specificity of these cells as reported previously [110, 116, 117]. In order to mimic OS that builds up prior to labor *in utero*, CSE has been utilized *in vitro* to treat AECs and AMCs [26].

Activation of p38MAPK by its increased phosphorylation was determined by WB in cell culture experiments. Similar to data reported in the intact fetal membranes above, we were able to replicate data reported earlier where CSE treatment caused an increased expression of P-p38MAP in both AECs (Fig. 3.2A)(p=0.005) as well as AMCs (Fig. 3.2B) (p=0.009) compared to cells grown under normal cell culture conditions (untreated control

cultures) [59, 110]. Activation of p38MAPK corresponded to inactivation of GSK3β as determined by the increased phosphorylation at the S9 site in response to CSE. Increased phosphorylation of GSK3β (S9) was noted in both AECs (Fig. 3.2C) (p=0.05) as well as AMCs (Fig. 3.2D) (p=0.04).

Interestingly, despite the inactivation of GSK3β, no change in the total β-catenin levels was noted in both AECs (Fig. 3.3A) (p=0.4) and AMCs (Fig. 3.3B) (p=0.4). β-catenin, being one of the most reported downstream targets of GSK3β, was expected to increase with increased phosphorylation of GSK3β as noted in other systems [29]. Nrf2, an antioxidant marker, another downstream target of GSK3β, was also analyzed. An increase in the relative levels of Nrf2 was noted in response to CSE in both AECs (Fig. 3.3C) (p=0.01) and AMCs (Fig. 3.3D) (p=0.005). Since both β-catenin and Nrf2 need to be translocated into the nucleus in order to perform their functions, we next determined the relative level of both molecules in the nuclear fraction of the cells in response to CSE treatment. The nuclear fraction of β-catenin was unchanged in response to CSE treatment confirming lack of its increase in response to CSE. However, nuclear translocation of Nrf2 was increased in response to the same treatment in AECs (Fig. 3.3E) (n=3) and AMCs (Fig. 3.3F) (n=3). To note, nuclear localization of Nrf2 was visibly higher in AMCs (Fig. 3.3F, middle panel) than AECs (Fig. 3.3E, middle panel). This is expected as AMCs are much more vulnerable and more pronounced in their response to OS than AECs. Han et al. had also recently reported a similar observation [118] where immortalized human amniotic mesenchymal cells (iHAMs) were more sensitive to oxidative damage compared to immortalized human amniotic epithelial cells (iHAEs).

Thus, CSE-induced inactivation of GSK3β increased the nuclear translocation of Nrf2 with no change in β-catenin levels in amnion cells. To verify p38MAPK-GSK3β axis in determining cell fate, we utilized p38MAPK inhibitor (SB203580) and GSK3 inhibitor (CHIR 99021) in order to confirm the upstream regulator and downstream target of GSK3β.

p38MAPK dependent inactivation of P-GSK3β in AECs and AMCs: Previous experiments correlated the phosphorylation of p38MAPK (activation) as well as GSK3β (inactivation) in response to CSE treatment in both AECs and AMCs. In order to determine if p38MAPK was, in fact, a regulator of GSK3β's function, a functional inhibitor to p38MAPK- SB203580 was tested. WB was performed and the relative expression of the proteins of interest were normalized densitometrically using associated β-actin levels in the samples (Supplemental Fig 3.1). Co-treatment of both AECs (Fig. 3.4A) ($p=0.05$) and AMCs (Fig. 3.4B) ($p=0.03$) with CSE and SB203580, caused a significant reduction in the Ser 9 phosphorylation of GSK3β. This suggested that p38MAPK was able to regulate the phosphorylation of GSK3β. Confirming our prior data, the relative level of β-catenin did not change in response to SB203580 treatment in AECs (Fig. 3.4A) ($p=0.4$) and AMCs (Fig.3.4B) ($p=0.2$). However, the expression of Nrf2 was blunted in response to SB203580 in AMCs (Fig. 3.4B) ($p=0.03$) and remained unchanged in AECs (Fig. 3.4A) ($p=0.1$).

In order to identify possible downstream targets of GSK3β, we utilized a potent pharmacological inhibitor of GSK3β-CHIR99021. In AMCs (Fig. 3.4D), CHIR99021 did not change the expression of either β-catenin ($p=0.2$) or Nrf2 ($p=0.1$). In AECs (Fig. 3.4C) however, the expression of both β-catenin ($p=0.08$) and Nrf2 ($p=0.08$) showed an increasing trend in response to treatment with CHIR99021. This suggested of independent effector mechanisms in AEC and AMC by GSK3β.

Cellular fate of β-catenin in amnion cells: Contrary to the expected increase of β-catenin when GSK3β is down regulated, we did not observe any increase either in the cytoplasm or in the nuclear translocation of β-catenin in response to CSE in amnion cells. Therefore, we hypothesized that β-catenin is exported out of the cells by packaging them in the exosomes. To test this, we isolated exosomes from media collected from AECs grown under normal conditions and AECs exposed to CSE. Isolated exosomes were confirmed using exosome marker CD81 and ZetaView PMX 110 (Supplemental Fig 3.2).

Interestingly, IF and confocal microscopy (Fig 3.5A, 3.5B) demonstrated increased colocalization of the exosome marker CD81 and β-catenin within the cytoplasm of CSE-treated AECs compared to controls. This was demonstrable by the line graph of the intensity of signal vs distance (Fig 3.5C) and analyzed by Pearson's correlation coefficient (Fig 3.5D) ($p<0.0001$) at the regions of interest within the cytoplasm of CSE treated AECs compared to control. The regions of interest at the membranes of control and CSE-treated AECs showed similar colocalization of CD81 and β-catenin as both proteins were present at the membranes [40, 119] (Fig 3.5C and 3.5D). The relative levels of β-catenin in exosomes from OS-induced cells were also higher than the control exosomes as demonstrated by WB (Fig 3.5E). Thus, β-catenin is possibly exported out of the cell in response to CSE preventing its nuclear translocation and its downstream function within the cell.

Cell fate in response to GSK3β inactivation: In order to understand the cell fate in response to inactivation of GSK3β, we used flow cytometry to identify changes in the cell cycle in AECs and AMCs. An increasing trend in the sub-G0G1 phase and a decrease in G2 phase was noted in response to the GSK3β inhibitor CHIR99021 in AECs (Fig. 3.6A) suggesting that inhibition of GSK3β could possibly cause cell cycle arrest in AECs. This was similar to the cell cycle arrest in response to CSE previously documented by our laboratory [52]. However, no changes in cell cycle were noted in AMCs (Fig. 3.6A) suggesting distinct mechanistic roles for GSK3β in AECs and AMCs.

Next, we performed flow cytometric SA–β-Gal assay to determine senescence induced by the GSK3β inhibitor CHIR99021. A significant increase in senescent cells compared to control was detected by C12FDG treatment in response to CHIR99021 in AECs (Fig. 3.6B) ($p=0.01$) and AMCs (Fig. 3.6C) ($p=0.04$). This finding was similar to what had been demonstrated earlier by our laboratory in response to CSE [59, 110]. This data suggested that inhibition of GSK3β can ultimately lead to senescence of amnion cells and overall this coincides with p38MAPK activation.

DISCUSSION

The primary goal of our study was to understand how OS and p38MAPK regulate GSK3β's function in the fetal membranes, particularly the amnion layer of the fetal membranes, and its possible contribution to the senescence phenotype. The effect of inducing OS on possible downstream targets of GSK3β that can determine cell fate, including β-catenin and Nrf2, were also analyzed. The key findings of our study are 1) TL is associated with activation of p38MAPK and inactivation of GSK3β. 2) In vitro, we recapitulated this data where OS conditions often seen at TL, induced active p38MAPK and inactive GSK3β in amnion membrane cells. Reduction of functional p38MAPK inhibited the phosphorylation of GSK3β, suggesting that p38MAPK can inactivate GSK3β. 3) Induction of OS on amnion cells was associated with an increased nuclear translocation of antioxidant Nrf2 with no change in pro-cell cycle mediator β-catenin but an increased secretion of β-catenin via exosomes. 4) Inhibition of GSK3β induced cell cycle arrest and senescence in amnion cells.

GSK3β is a constitutively expressed pro-cell cycle protein during gestation and parturition. In reproductive tissues, GSK3β has been shown to play a role in survival, differentiation, and migration of placental trophoblast cells [120-122]. Interestingly, evidence that points to the contribution of GSK3β in promoting a seemingly opposing function of cell senescence also exists [123, 124]. Seo et al. linked GSK3 inhibition to enhanced glycogenesis and cell senescence [125]. Similarly, Kim et al. demonstrated that inactivation of both isoforms of GSK3 can cause an increase in lipogenesis and subsequent senescence of cells [126]. The dual role of GSK3β is possibly determined by the cell type, the stimulus, and phosphorylation of a specific site by its upstream regulator. Thus, understanding the exact functional and mechanistic role of GSK3β in the tissue of interest is a challenge. The systematic review conducted by us identified important knowledge gaps

in studying mechanisms and functions involving GSK3β in reproductive tissues [29]. One of the major knowledge gaps identified was that the role of p38MAPK in regulating GSK3β's function in reproductive tissues in general, and during parturition in particular, has not been studied [29]. As far as we know, this is the first study in fetal membranes to establish the link between p38MAPK and GSK3β.

The role of p38MAPK in regulating GSK3β has been reported previously by Thornton TM et al. [105]. In thymocytes, the authors reported a p38MAPK dependent inactivation of GSK3β leading to an accumulation of β-catenin [105]. In these cells, p38MAPK-mediated phosphorylation of GSK3β was at T390 [105]. This was confirmed in GSK3β Thr390 mutant cells where p38MAPK mediated phosphorylation was partially abrogated [105]. Our experimental conditions and T390 specific antibody did not demonstrate GSK3β phosphorylation in either AECs or AMCs (data not shown). A similar phenomenon has been observed in AECs and AMCs with p38MAPK phosphorylation where these cells tend to have distinct mechanisms of activation where they do not exhibit canonical apoptosis signal-regulating kinase (ASK)-1 mediated activation of p38MAPK and are often activated by TGFβ-TAB1 mediated autophosphorylation [104]. Gene silencing of TAB1 prevented p38MAPK activation whereas ASK1 inhibition did not impact p38MAPK [104]. Lack of GSK3β phosphorylation at T390 but at S9 is also unique in these cells type under OS conditions induced by CSE. The uniqueness of activation and inactivation of various molecules related to cell growth and senescence is assuring timed events taking place during gestation and parturition. Canonical activation of p38MAPK can be detrimental to GSK3β mediated feto-placental growth and survival during pregnancy. However, OS mediated increase of TGFβ in the amniotic fluid and amniochorion assures, p38MAPK activation that will promote GSK3β inhibition at a definite site to ensure senescence and inflammation required for timely delivery of the fetus. We postulate that unlike other cells; human fetal membrane cells exhibit unique pathways to regulate signaling molecules' functions to ensure pregnancy survival as well

as determine longevity of fetal membranes. This is a natural and physiologic response from the membranes as their functional role as protector of uterine cavity is over. In contrast, redox balance during pregnancy prevents p38MAPK activation and maintains active GSK3β to perform proliferative functions in amnion membrane cells to help them remodel during pregnancy to maintain their integrity and function. Senescence and sterile inflammation from senescent cells, a non-reversible process, eventually promotes parturition by muting GSK3β function and transporting β-catenin outside the cell.

Our study analyzed a possible downstream target of p38MAPK- GSK3β. Like p38MAPK, GSK3β is a multifunctional kinase that regulates multiple biological processes within the cell [29, 30, 127, 128]. As an integral component of the Wnt pathway or the PI3K/AKT pathways, GSK3β and its downstream target β-catenin have played an important role in cellular proliferation, cell survival and embryonic development [60, 129, 130]. β-catenin is one of the most studied downstream targets of GSK3β [29]. Amongst other functions, β-catenin is a pro-growth factor that helps maintain the pluripotency of stem cells [131, 132]. An increase in the nuclear translocation of β-catenin by abnormal activation of the Wnt pathway has been noted to play a major role in the development and progression of tumors [133]. Interestingly, some studies highlight the specific role of β-catenin in bypassing cellular senescence [134, 135]. In our study, the expression of β-catenin was unchanged in TL fetal membranes compared to TNIL samples. Further, treatment of AECs and AMCs with either CSE or CHIR99021 did not change the relative levels of β-catenin. Also, no nuclear translocation of β-catenin was noted in response to CSE. These findings suggested that OS causes inactivation of GSK3β in amnion cells possibly does not involve the Wnt/β-Catenin pathways.

Further, a higher relative level of β-catenin was packaged within exosomes derived from OS-induced AECs compared to AECs grown under normal conditions and exported out of the cells. Exosomes carrying specific cargo can function as either ‘garbage bags’ to remove unwanted molecules from the cell or can utilize the cargo as signaling molecules

and a means of intercellular communication [53, 56]. We speculate that OS in AECs possibly suppresses the cell proliferative actions of the Wnt/ β-catenin signaling pathway by removing any β-catenin that has been freed from the β-catenin destruction complex and packaging it within exosomes.

Fetal membranes perform important biological functions during gestation; however, as discussed above, their need is limited to this specific period of time. It is thus possible that fetal membrane cells promote senescence by removing excess β-catenin from the cells under conditions of OS, as noted towards the end of the gestational period. Hoffmeyer et al. have demonstrated that β-catenin deficient mouse embryonic stem cells have reduced telomerase activity and shortened telomeres [136]. OS-induced telomere length loss has been noted in fetal membranes [23]. Cell-free fetal telomere fragments arising from fetal membranes are shown to get packaged in AEC derived exosomes as well as accumulate in amniotic fluid prior to delivery at term [114]. Removal of β-catenin within exosomes under OS conditions to indirectly promote reduction of telomere length of amnion cells along with the senescence phenotype is a novel possibility. Future mechanistic studies planned by our laboratory will help determine the role of β- catenin present within exosomes derived from OS-induced AECs.

Although CSE is able to induce OS and p38 MAPK activation in amnion cells *in vitro*, the response of amnion cells to CSE may not be extrapolated to infection during pregnancy associated with preterm birth. We have reported increased numbers of senescent amnion cells without p38 MAPK activation in response to treatment with lipopolysaccharide (LPS) and TNF-α to mimic changes associated with infection and inflammation [59]. Although both LPS and TNF-α induce p38 MAPK-mediated senescence, the extent of senescence was different than CSE. Further, preliminary studies conducted by our laboratory (data not shown) did not show any change in the phosphorylation of GSK3β in AECs in response to LPS treatments. It is likely that the pathways associated with LPS/infection induced amnion cell senescence may bypass p38

MAPK/ GSK3β associated signaling. Distinct mechanisms of activation of p38MAPK and inflammatory pathways by infectious and non-infectious mediators have also been reported previously by us supporting this postulation [137].

Another downstream target of GSK3β is Nrf2 [107]. In fetal membranes, Nrf2 has been demonstrated to have anti-inflammatory properties [138]. In the present study, we noted that CSE was able to increase the relative levels of Nrf2 in amnion cells. The nuclear translocation of Nrf2 in response to CSE was also demonstrable. Pharmacological inhibition of p38 MAPK and GSK3β gave inconclusive data regarding the regulation of Nrf2 by p38MAPK or GSK3β. This could be due to insufficient power required for experiments using these inhibitors. Gene silencing and overexpression studies have been planned by our laboratory to better establish the possible role of Nrf2 in OS-induced amnion membrane senescence.

Treatment of amnion cells with CSE has been demonstrated to induce cell cycle arrest and senescence of the cells in previous studies conducted by our laboratory [52, 59]. In the present study, we noted that inhibition of GSK3β by CHIR99021 induced senescence as well which was comparable to that caused by CSE. Further, the treatments induced an arrest in cell proliferation in AECs. Thus, senescence of the amnion cells involves the activation of p38MAPK and the inactivation of GSK3β.

In conclusion, our present study demonstrates the novel regulation of GSK3β by p38MAPK in fetal membrane cells (Fig. 3.7). OS causes the activation of p38MAPK and the inactivation of GSK3β. This signaling mechanism can contribute to the senescence of amnion membrane cells. Further mechanistic studies are needed to determine if senescence of amnion cells can possibly be mediated by Nrf2, which has been recently demonstrated to occur in fibroblasts [139]. Nrf2 has also been shown to potentiate the actions of Peroxisome proliferator-activated receptor ϒ (PPAR-γ) and cause the inhibition of the Wnt/β-catenin pathway [140-143]. Understanding the role of GSK3β in the oxidative stress response and senescence of fetal membranes will help determine if it is a potential target

to develop therapeutic strategies to minimize damage associated with OS during pregnancy including PTB.

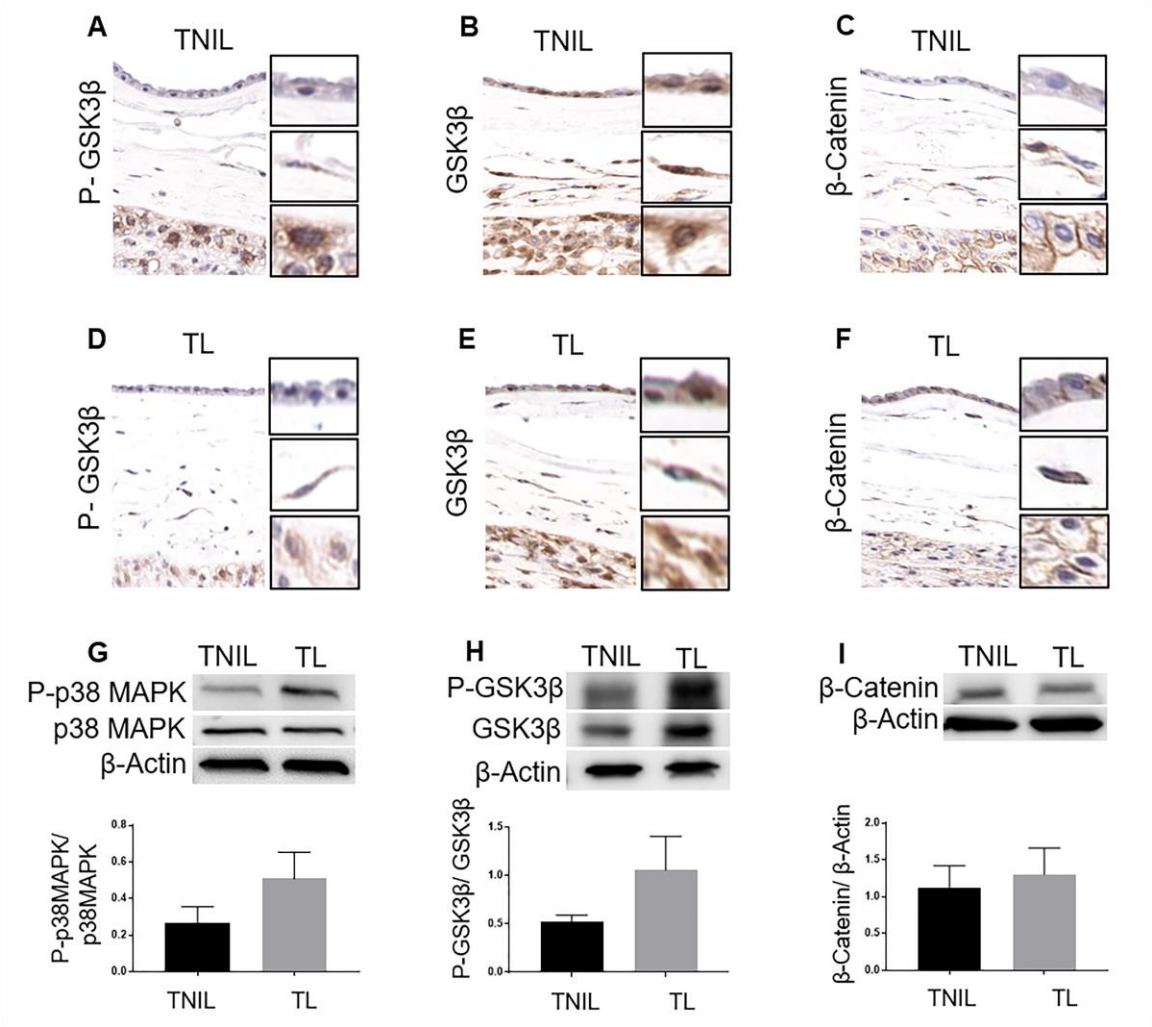


Fig. 3.1 Localization and expression of GSK3β and β-Catenin in fetal membranes

A, D: Immunohistochemistry (IHC) images of term not in labor (TNIL) and term labor (TL) fetal membranes stained for P-GSK3β.
B, E: IHC images of TNIL and TL fetal membranes stained for Total GSK3β.
C, F: IHC images of TNIL and TL fetal membranes stained for β-Catenin.
Representative images from a minimum n=3 for each tissue type are shown. P-GSK3β, Total GSK3β and β-Catenin were found localized to both amnion and chorion layers of the fetal membrane.
G: Western blots (WB) of P-p38MAPK in TL vs. TNIL fetal membranes.
H: WB of P-GSK3β in TL vs. TNIL fetal membranes.
I: WB of β-Catenin in TL vs. TNIL fetal membranes.
The relative levels of P-p38MAPK and P-GSK3β increased two-fold in TL compared to TNIL samples. Total p38MAPK, total GSK3β, and total cellular β-Actin were used for normalization (in G, H and I respectively). Representative blots from an n=12 for each tissue type are shown. Data are presented as mean ± SEM.

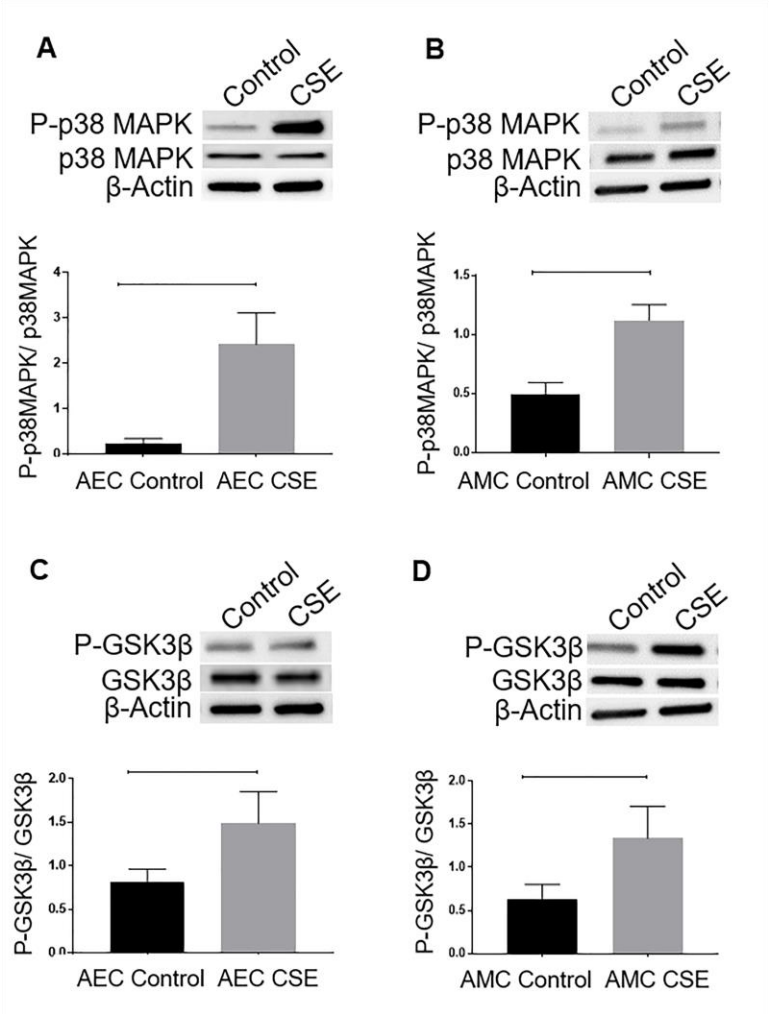


Fig. 3.2 Effect of CSE on p38MAPK and GSK3β expression in the amnion membrane.

A-B: WBs of amnion epithelial cells (AECs) and amnion mesenchymal cells (AMCs) for P-p38MAPK in response to cigarette smoke extract (CSE) treatment.
C-D: WBs of P-GSK3β in AECs and AMCs in response to CSE treatment.
The relative levels of P-p38MAPK and P-GSK3β significantly increased in response to CSE treatment compared to controls in both AECs ($p=0.005$ and $p=0.05$ respectively) and AMCs ($p=0.009$ and $p=0.04$ respectively). Total p38MAPK and total GSK3β was used for normalization. Representative blots from a minimum n=3 for each cell type are shown. Data are presented as mean ± SEM.

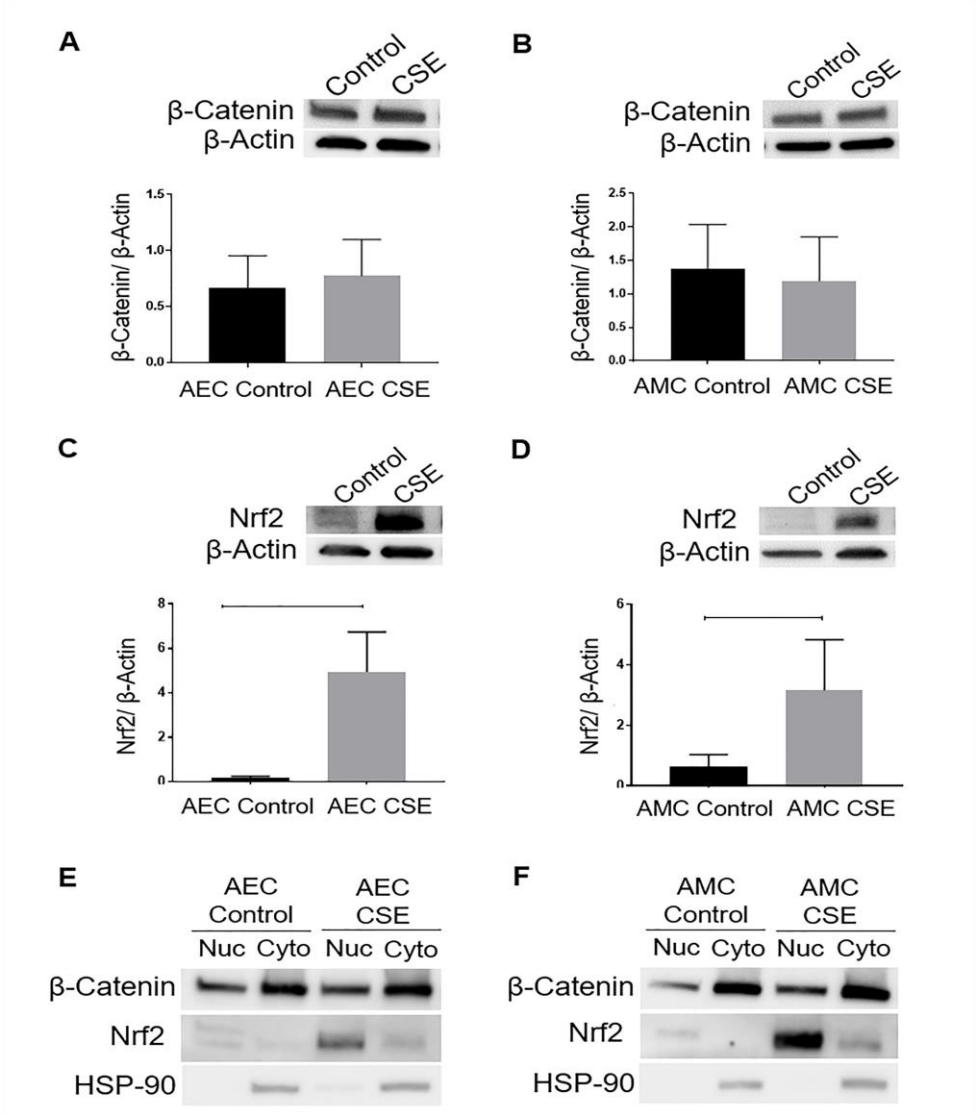


Fig. 3.3 Effect of CSE on possible GSK3β downstream targets in the amnion membrane

A-B: WBs of AECs and AMCs for β-Catenin in response to treatment with CSE.
C-D: WBs of AECs and AMCs for Nrf2 in response to treatment with CSE.
The relative levels of Nrf2 significantly increased in response to CSE treatment compared to controls in both AECs and AMCs ($p=0.01$ and $p=0.005$ respectively). Total cellular β-Actin was used for normalization. Representative blots from a minimum n=3 for each cell type are shown. Data are presented as mean ± SEM.
E: Western blot analysis of nuclear (Nuc) and cytoplasmic (Cyto) lysates demonstrates increased nuclear translocation of Nrf2 in response to CSE with no change in the level of β-Catenin in AECs
F: Western blot analysis of Nuc and Cyto lysates demonstrates increased nuclear translocation of Nrf2 in response to CSE with no change in the level of β-Catenin in AMCs. Heat shock Protein 90 (HSP-90) was used to confirm the purity of extraction of nuclear and cytoplasmic proteins. Representative blots from a minimum n=3 for each cell type are shown.

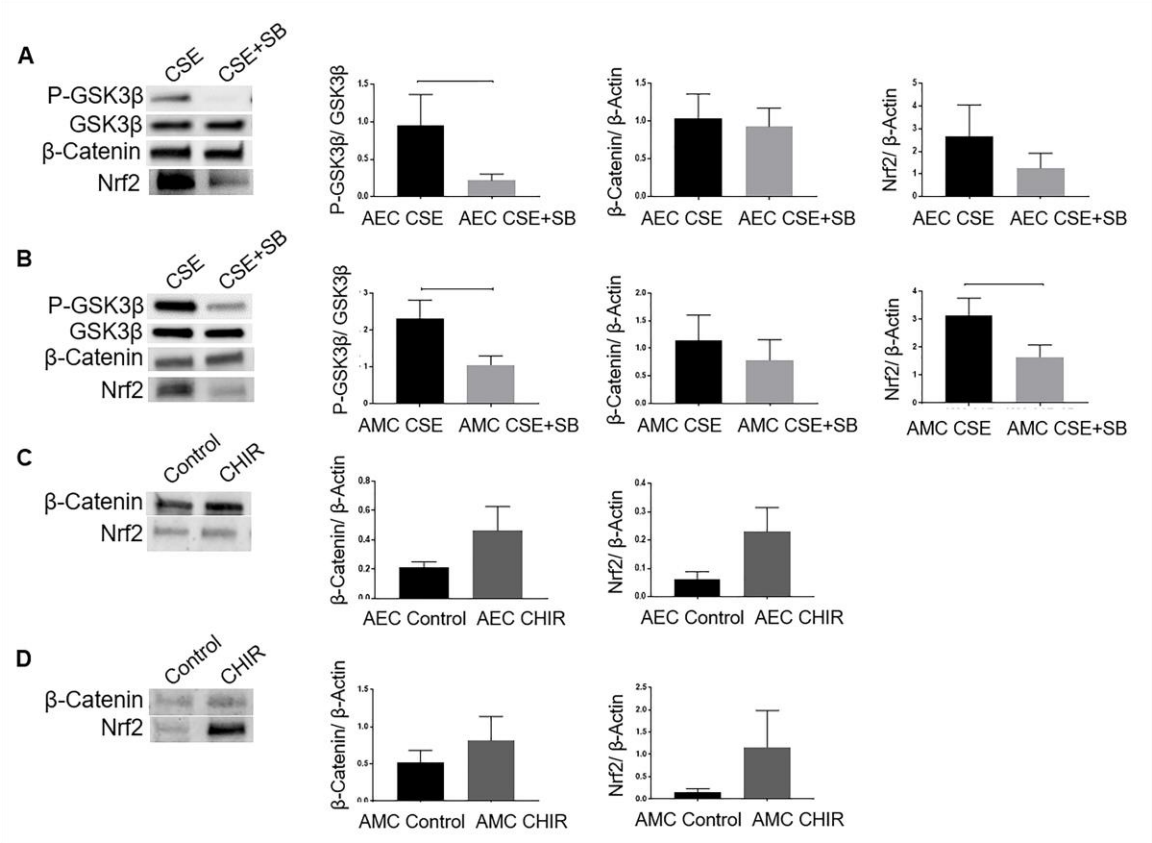


Fig. 3.4 p38MAPK dependent inactivation of P-GSK3β in AECs and AMCs

A: WBs of CSE and CSE+SB203580 (SB) treated AECs for P-GSK3β, total GSK3β, β-catenin and Nrf2.

B: WBs of CSE and CSE+SB treated AMCs for P-GSK3β, total GSK3β, β-catenin and Nrf2.

The relative levels of β-Catenin were unchanged (AECs and AMCs) while that of Nrf2 was attenuated (AMCs) in response to treatment with SB. Total GSK3β was used for normalization.

C: WBs for β-Catenin and Nrf2 in response to GSK3β inhibitor CHIR99021 (CHIR) in AECs.

D: WBs for β-Catenin and Nrf2 in response to GSK3β inhibitor CHIR in AMCs. Total cellular β-Actin was used for normalization.

The relative levels of β-catenin or Nrf2 remained unchanged in AMCs. In AECs however, the expression of both β-catenin ($p=0.08$) and Nrf2 ($p=0.08$) showed an increasing trend in response to treatment with CHIR. Representative blots from a minimum $n = 4$ for each cell type are shown. Data are presented as mean ± SEM.

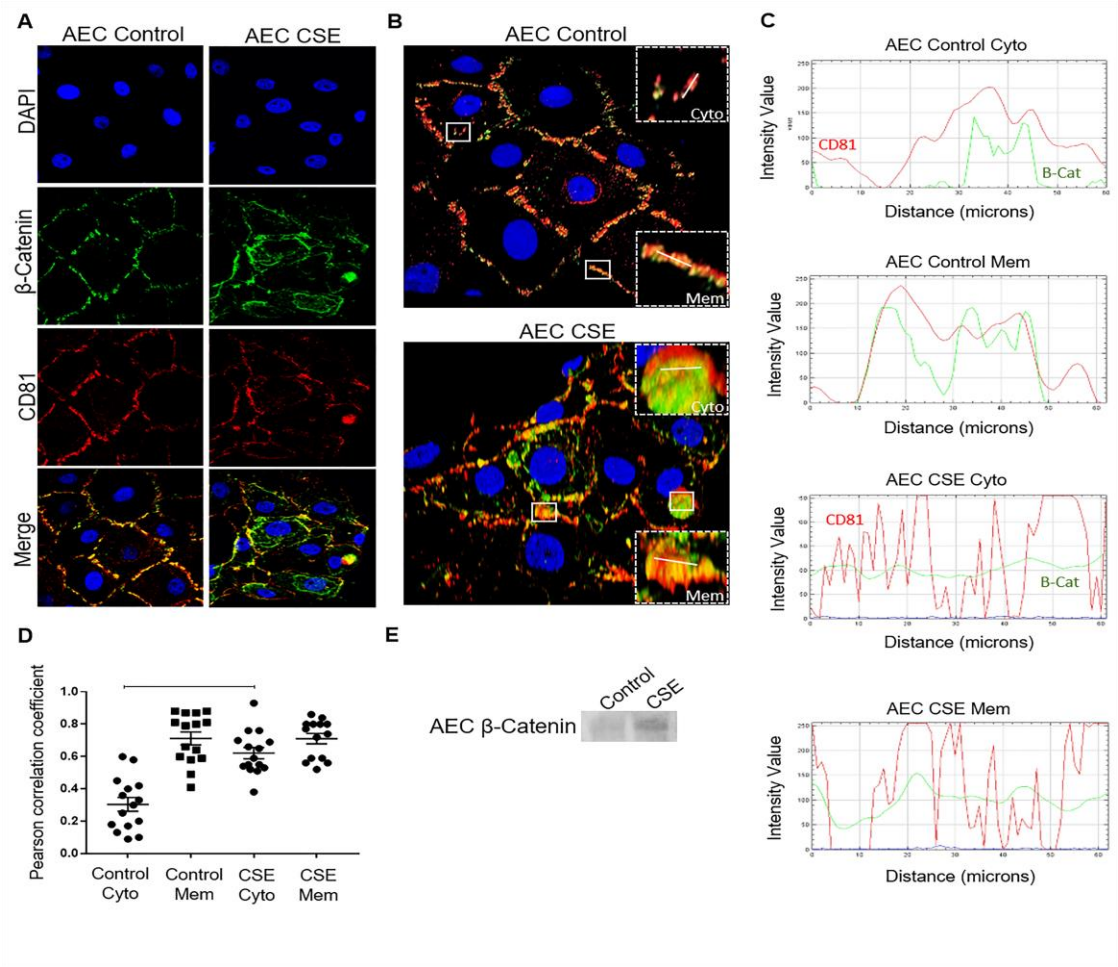


Fig. 3.5 Fate of β-Catenin in AECs.

A: Confocal microscopy of control and CSE treated AECs showing β-Catenin (green) and CD81 (red) localization. CSE treatment induced cytoplasmic β-Catenin and CD81 compared to controls.

B: 3 dimensional confocal images highlighting membrane (Mem) and cytoplasmic (Cyto) region of interest (crops; n=15) selected for co-localization analysis. Control AEC (top) CSE treated AEC (bottom). White lines represent 60μM line graph region of interest. DAPI –nuclear stain (blue); β-Catenin (green); CD81 – exosome marker (red).

C: The line graphs demonstrate overlap between CD81 and β-catenin signal at the region of interest at the cytoplasm (Cyto) and membranes (Mem) of both control and CSE treated AECs. CSE exosomes contained more β-catenin-CD81 overlap than control exosomes.

D: The Pearson's correlation coefficient showed significantly higher co-localization at the regions of interest in the cytoplasm of CSE treated AECs compared to controls ($p<0.0001$). Data are presented as mean ± SEM.

E: WB analysis of relative levels of β-catenin within AEC exosomes. Representative blot from a minimum of n=3 is shown. OS increased β-catenin in exosomes compared to exosomes from cells cultured under normal conditions.

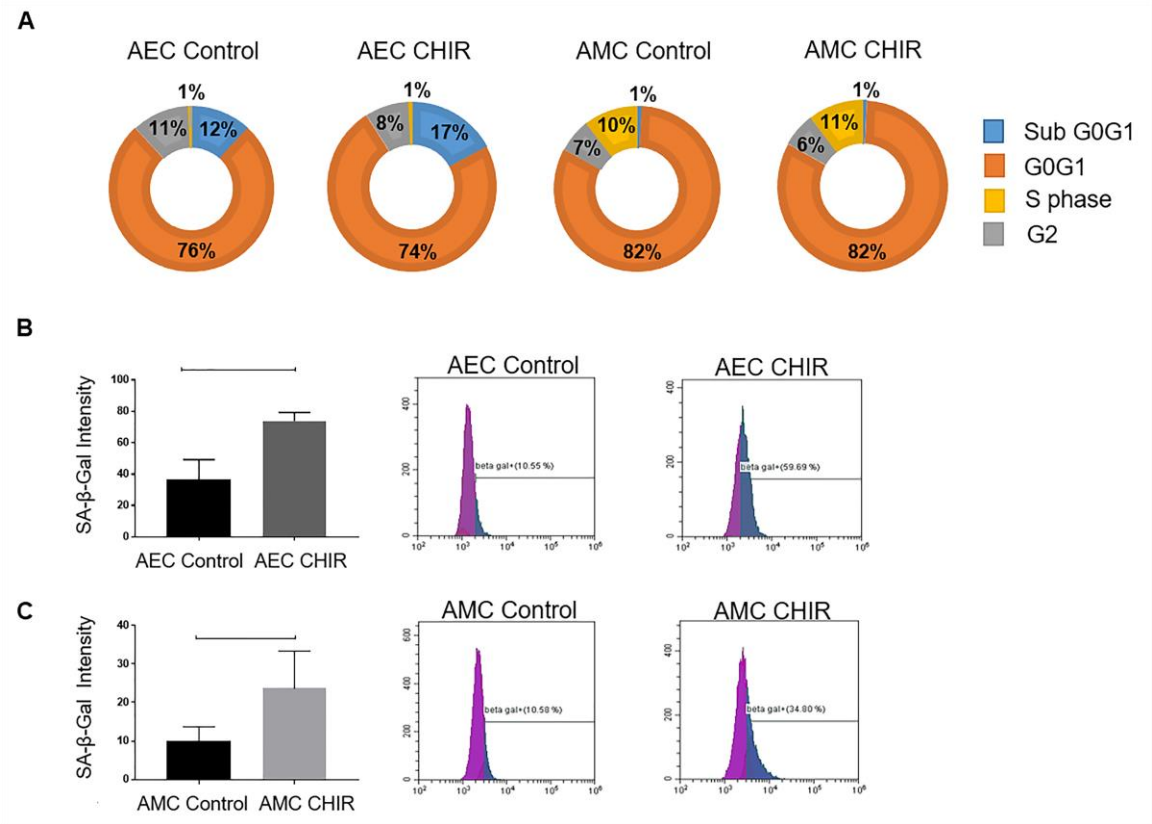


Fig. 3.6 Cell fate in response to GSK3β inactivation

A: Flow cytometric cell cycle analysis of AECs and AMCs in response to treatment with control medium and CHIR. CHIR caused an increase in percent of cells in sub-G0G1 phase and reduced those in G2 phase in AECs compared to control.
B-C: Flow cytometric analysis of senescence (SA-β-Gal assay) of AECs and AMCs in response to treatment with CHIR compared to control. A significant increase in percent of senescent cells was noted following treatment in both AECs (p=0.01) and AMCs (p=0.04) compared to controls. Representative data from a minimum n=3 for each cell type are shown. Data are presented as mean ± SEM.

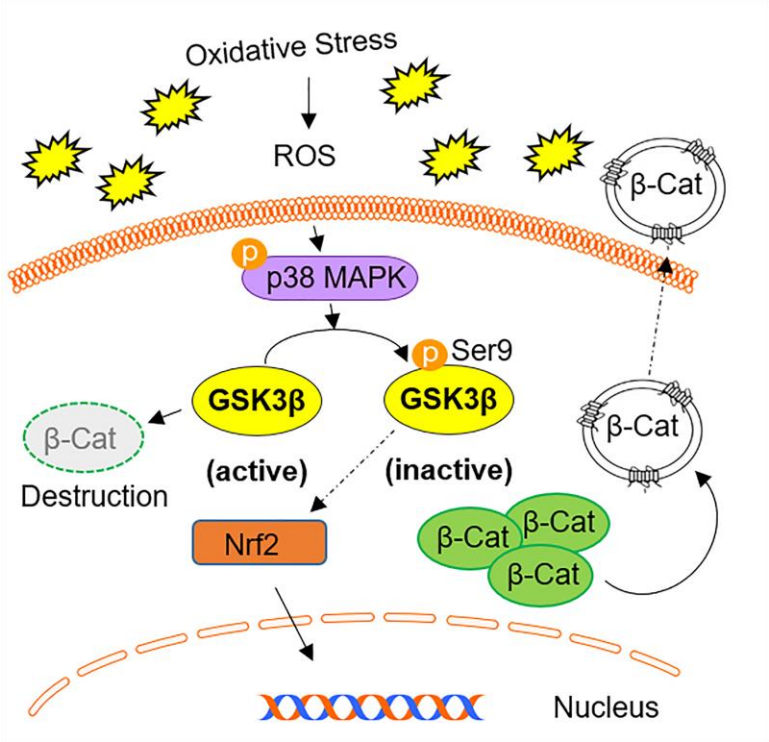


Fig. 3.7 Summary figure representing the proposed pathway of OS induced p38MAPK-GSK3β mediated signaling in amnion cells.

OS-induced reactive oxygen species (ROS) activates p38MAPK and mediates the inactivation of GSK3β by phosphorylating Ser9. Inactivation of GSK3β effects downstream targets Nrf2 and β-catenin (β-Cat). In response to OS, there is increased translocation of Nrf2 into the nucleus likely as an antioxidant response. Active GSK3β degrades β-catenin, while OS-induced GSK3β inactivation allows for the accumulation of β-catenin in the cell. AEC β-catenin does not get translocated to the nucleus; instead, β-catenin is likely packaged within exosomes and cargoed out of the cell.

CONCLUSIONS

Chapter 4. Summary and future directions

THESIS SUMMARY

OS has been linked to the pathogenesis of multiple human diseases including cancer, cardiovascular disorders, like atherosclerosis and hypertension, neurological diseases, like Parkinson's and Alzheimer's disease, as well as respiratory and renal disorders [144]. OS has been shown to contribute to normal and abnormal labor mechanisms as well [23].

An increase in OS at the time of parturition can be attributed to (i) An increase in the metabolic demands of the feto-maternal unit and increased production of ROS, with reduced availability of antioxidants (ii) An inflammatory overload, with an increase in prostaglandins and cytokines (e.g. IL-6, TNF α), leading to the production of ROS [59, 145]. Our laboratory has demonstrated how increased OS can cause telomere shortening and p38MAPK activation which contribute to the senescence of fetal membranes and ultimately labor and delivery of the fetus [23, 24, 146]. A similar mechanism can be triggered by OS prematurely, contributing to PTB [4, 58]. It is thus, important to understand the mechanisms underlying OS stress induced labor pathways. Identification of possible therapeutic targets can help curtail high PTB rates and improve maternal and fetal morbidity and mortality.

GSK3β is a potential downstream target of p38MAPK that contributes to senescence of fetal membranes. The regulation of GSK3β by p38MAPK has been demonstrated in other fields [101, 105]. In an attempt to understand the functional role of GSK3β in pregnancy and parturition before conducting our study, we undertook a systematic review of literature. The systematic review revealed that GSK3β plays

important functional roles in reproductive tissues right from implantation of the blastocyst up to delivery of the fetus [29]. The role of GSK3β in important complications of pregnancy including preeclampsia, gestational diabetes mellitus and fetal growth restriction have been elucidated [29]. The potential role of GSK3β in causing preterm labor has also been explored. Further, GSK3β may be regulated by multiple upstream regulators and may be a part of important pathways within the cell including the PI3K/AKT pathway, Wnt pathway as well as ROS induced pathways [29]. However, the regulation of GSK3β by p38MAPK specifically in reproductive tissues has not been explored.

Having understood that GSK3β is a multifunctional kinase and part of multiple important signaling pathways in the cell, with a potential role to play during labor- both term and preterm, we attempted to explore the contribution of GSK3β to OS induced senescence of fetal membranes. Our experiments helped us conclude that signaling mechanisms in the fetal membranes at term are associated with an activation of p38MAPK and inactivation of GSK3β. Further, OS induced *in vitro* in amnion cells using CSE caused a similar activation of p38MAPK and inactivation of GSK3β. The regulation of GSK3β by p38MAPK was confirmed with the help of a functional inhibitor of p38MAPK SB203580. The effect of OS induction in amnion cells on two downstream targets of GSK3β, antioxidant Nrf2 and pro-cell cycle mediator β-catenin, were also explored. We demonstrated that OS induction caused an increase in nuclear translocation of Nrf2 with no change in the nuclear levels of β-catenin. Interestingly, an increased secretion of β-catenin via exosomes was demonstrable. Inhibition of GSK3β overall induced cell cycle arrest and senescence in amnion cells.

Therefore, future studies will test the hypothesis that during pregnancy, p38MAPK activation in fetal membranes is kept under check. However, as pregnancy advances and there is increased OS induction within the intra uterine compartment, fetal membranes senesce due to an activation of p38MAPK and inactivation of GSK3β. This phenotype may be further promoted by increased transport of β-catenin outside the cell packaged in

exosomes. Overall, this mechanism may contribute to delivery of the fetus along with the fetal membranes.

SIGNIFICANCE

Our research helped reveal a novel pathway that maybe regulating labor. The results from our studies help bridge important gaps in knowledge in the field of parturition and helped identify the interaction between multifunctional kinases like GSK3β and p38MAPK, as they relate to amnion membranes. GSK3β has been reported to be involved in various biological processes in reproductive tissues. However, specific role of GSK3β and the role of GSK3β's downstream targets like Nrf2 and β-catenin, in the amnion membrane, has been explored for the first time. Our studies helped establish the unique role of GSK3β in causing senescence of amnion cells in the amnion membranes at the time of labor.

Further, for the first time, we have examined a possible role of exosomes in contributing to the senescence of amnion cells. Thus, our project helped establish the singular concept of propagation of signals via exosomes in amniotic membranes, during labor, and represent an important contribution in this field.

FUTURE DIRECTIONS

The results from our studies helped identify a specific signaling pathway that may contribute to senescence of the amnion membrane. This knowledge will help develop novel therapeutic strategies to prevent premature senescence of fetal membranes that contributes to preterm labor. Based on the findings and conclusions from our study, the Menon lab will continue to explore the contribution of GSK3β to premature labor. Gene silencing studies will be undertaken to further confirm the findings of our study and identify novel downstream targets of GSK3β that may also serve as possible therapeutic targets.

As it has been established that senescence of fetal membranes may be associated with the removal of β-catenin within exosomes, the treatment of cells with bio-engineered β-catenin containing exosomes to prevent the senescent phenotype from developing, will be explored. Packaging drugs within exosomes is a novel therapeutic strategy which may be made use of in clinical medicine to reduce the incidence of preterm birth.

Experiments to explore the utility of Nrf2 induction in fetal membrane cells are also being undertaken in the Menon lab. Sulforophane is an inducer of the anti-oxidant Nrf2. Treatment of amnion cells with sulforophane to prevent the development of senescence will be explored in future experiments. The ultimate goal of the proposed experiments is to help devise a therapeutic strategy to prevent the development of premature senescence of fetal membranes and subsequently preterm labor.

Appendix

A.

Quality Assessment	Yes (Score: 2)	Partial (Score: 1)	No (Score: 0)	N/A
1) Question/ Objective sufficiently described?				
2) Design approporiate to answerstudy question?				
3) Samples, reagents, assays sufficiently described?				
4) Methods described in sufficient detail?				
5) Study can be easily replicated?				
6) Sample/ Experiment number sufficient for internal validity?				
7) Appropriate negative controls?				
8) Appropriate statistical analysis?				
9) Results reported in sufficient detail?				
10) Do the results support the conclusion?				
Total score				

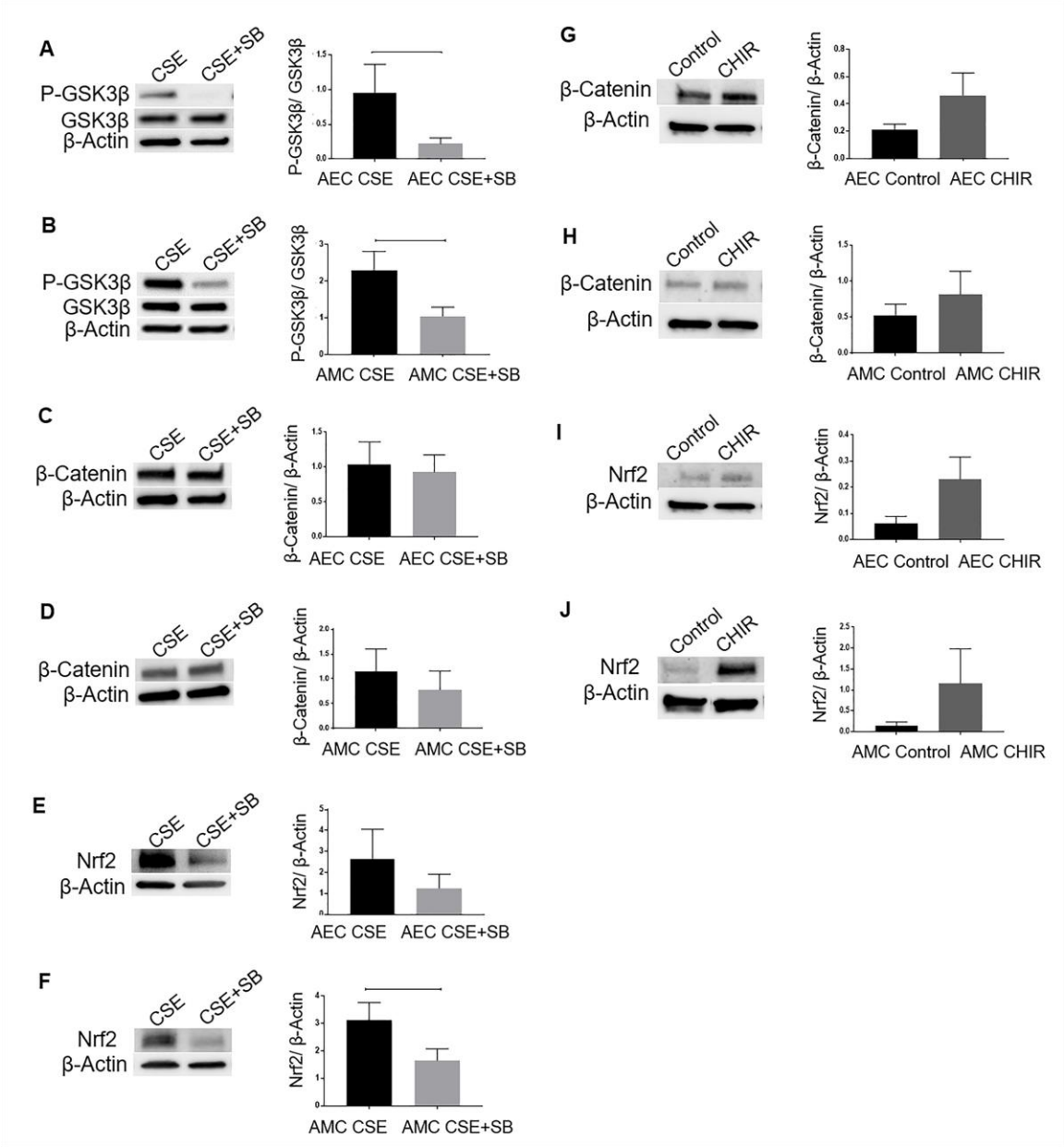
B.

	Number of good quality papers	Number of acceptable quality papers	Number of poor quality papers
Q1.	21	4	0
Q2.	21	4	0
Q3.	22	3	0
Q4.	23	1	0
Q5.	23	1	0
Q6.	19	6	0
Q7.	23	1	0
Q8.	19	6	0
Q9.	24	0	0
Q10.	23	1	0

Sup. Fig. 2.1 Criteria for quality assessment of selected articles for review.

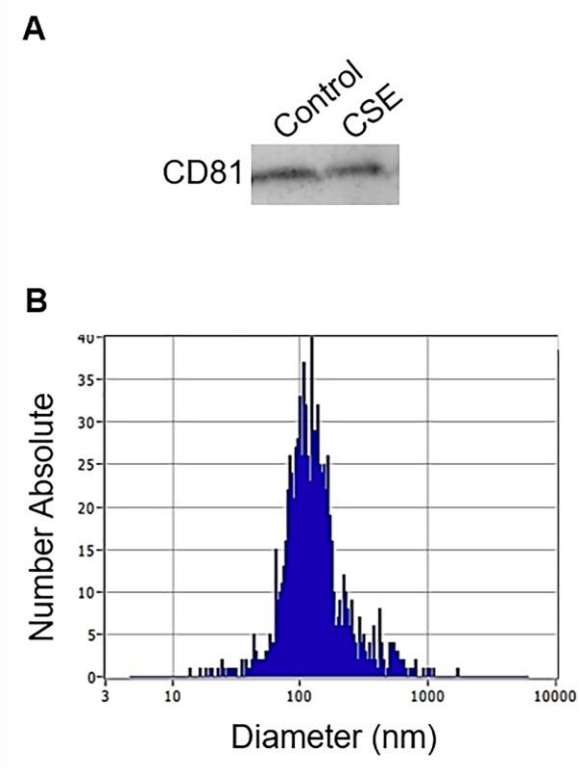
A. Table depicting the questions/ criteria used for quality assessment of the selected articles.

B. Table depicting the number of good/ acceptable/ poor quality papers included in the final review for each of the defined criteria.



Sup. Fig. 3.1 p38MAPK dependent inactivation of P- GSK3β in AECs and AMCs

A-F: WB analysis of CSE treated AECs and AMCs for P-GSK3β (A-B), β-Catenin (C-D) and Nrf2 (E-F) in response to co-treatment with SB203580 (SB) presented as mean ± SEM. Total GSK3β and total cellular β-Actin were used for normalization.
G-J: WB analysis of AECs and AMCs for β-Catenin (G-H) and Nrf2 (I-J) in response to GSK3β inhibitor CHIR99021 (CHIR) presented as mean ± SEM. Total cellular β-Actin was used for normalization (n=4).



Sup. Fig. 3.2 Confirmation of isolated exosomes' identity

A: Representative WB depicting the exosomal marker CD81 for control and CSE treated AEC exosomes.
B: Zetaview analysis graph confirming the size of the isolated AEC exosomal particles.

Curriculum Vitae

NAME: **Narmada Lavu**

PRESENT POSITION: Graduate Assistant
The University of Texas Medical Branch at Galveston
Department of cell biology
301 University Boulevard
Galveston, Texas 77555-1061

BIOGRAPHICAL: Born: Hyderabad, India
Current Home Address: 6315 Central City Blvd,
Apt #120, Galveston, Texas-77551
Phone: 216-446-9189 (cell)
E-mail: nalavu@utmb.edu

EDUCATION:

- Graduate Student
  Department of Cell Biology
  The University of Texas Medical Branch at Galveston
  August 2017 – Present

- Post-Graduate Medical Education (Residency)
  Obstetrics and Gynecology (M.S.-Master of Surgery),
  Kasturba Medical College, Mangaluru,
  Manipal University, Karnataka, India.
  May 2012 – May 2015

- Medical school
  Bachelor of Medicine and Bachelor of Surgery,
  Deccan College of Medical Sciences,
  Hyderabad, India.
  July 2006-March 2012

PROFESSIONAL EXPERIENCE:

- Andrologist
  Centre of Reproductive Medicine, Webster (Texas)
  February 2017-July 2017

- Physician
  Anu Test Tube Baby Centre, Hyderabad (India)
  November 2015-April 2016; October 2016-November 2016

- Intern at clinical embryology laboratory
  Anu Test Tube Baby Centre, Hyderabad (India)
  November 2015-April 2016; October 2016-November 2016

TEACHING EXPERIENCE:

- Guest Lecturer: UTMB High School Summer Biomedical Research Program, July 2018

- Guest speaker: UTMB summer STEM camp (for 8th and 9th grade students), June 2018.

- Clinical instructor: Clinical teaching and case discussions (Obstetrics and Gynecology) for final year medical students
  Kasturba Medical College,
  December 2012-December 2014

- Clinical instructor: Clinical case discussions for medical (3rd year and final year) students.
  Deccan college of medical sciences
  February 2011- March 2012

- Tutoring first year medical students in pre-clinical subjects:
  Deccan college of medical sciences
  August 2007-2009

RESEARCH ACTIVITIES:

RESEARCH INTERESTS: I am interested in Obstetrics and Gynecology based research. Currently, my research focuses on signaling pathways in the placental membranes leading to term and preterm labor.

Post Graduate Thesis:

Title: Serum B-hCG and Lipid Profile in early second trimester as a predictor of hypertensive disorders of pregnancy.

Mentor: Dr.Vijaya M.Revankar, Associate professor,
Department of Obstetrics and gynecology,
Kasturba Medical College, Manipal University, India.
Study period: November 2012-May 2014

Publications:

- ***Lavu,N.***, Radnaa,E., Kechichian,T., Richardson,L., Sheller-Miller,S., Bonney,E., Menon,R. (2019). ***Oxidative Stress Induced Downregulation of Glycogen Synthase kinase 3β (GSK3β) in Fetal Membranes Promotes Cellular Senescence.*** Biolre (accepted).

- ***Lavu,N.***, Richardson,R., Bonney,E., Menon,R. (2018). ***Glycogen synthase kinase (GSK) 3 in pregnancy and parturition: a systematic review of literature.*** J Maternal Fetal Neonatal Med, 1-221.

- *Revankar V.M. &* ***Lavu,N. (2017). Assessment of Serum B-hCG and Lipid Profile in early second trimester as predictors of hypertensive disorders of pregnancy.*** Int J Gynecol Obstet, 138(3), 331-334. doi:10.1002/ijgo.12225.

- *(Abstract)* ***Lavu, N.***, Lavu, D., Uppal, H., Chandran, S., & Potluri, R. (2016). ***Shorter length of hospital stay following hysterectomy in Afro-Caribbeans***. BJOG: Int J Obstet Gy, 123(Suppl S2).

- ***Lavu, N.***, Revankar, V., Rao, A., Lavu, D., & Potluri, R. (2014). ***Trends and Rates of Caesarean Sections: Seven Year Study in Mangalore.*** *International Journal of Innovative Research And Development, 3*(8).

- *(Abstract)* ***Lavu, N***., Uppal, H., Rao, A. A., Lavu, D., & Potluri, R. (2013). ***Predictors of mortality in patients with cervical cancer-A hospital admissions study: 2000-2012.*** International Journal of Gynecological Cancer, 8 (Suppl 1).

Presentations:

Oral:

- ***Caesarean Sections: Seven Year Trends***.
  Narmada Lavu, Vijaya M Revankar, A.Arun Rao, Deepthi Lavu
  Yuva FOGSI (Federation of Obstetric and Gynecological Societies of India)-South Zone, Mysore, India, June 2014.

Posters:

- ***Oxidative Stress Induced Downregulation of Glycogen Synthase kinase 3β (GSK3β) in Fetal Membranes Promotes Cellular Senescence***
  Narmada Lavu, Enkhutaya Radnaa, Talar Kechichian, Lauren Richardson, Samantha Sheller-Miller, Elizabeth Bonney, Ramkumar Menon
  Cell Biology symposium, UTMB (Galveston), April 2019.

- ***Role of GSK3 (Glycogen Synthase Kinase 3) in pregnancy and parturition: A systematic review of literature***
  Narmada Lavu, Lauren Richardson, Ramkumar Menon
  Cell Biology symposium, UTMB (Galveston), May 2018.

- ***Role of GSK3 (Glycogen Synthase Kinase 3) in pregnancy and parturition: A systematic review of literature***
  Narmada Lavu, Lauren Richardson, Ramkumar Menon
  The Center for Interdisciplinary Research in Women's Health: Poster Session for women's health research, UTMB (Galveston), May 2018. **Awarded 2nd place for best basic science research poster.**

- ***Shorter length of hospital stay following hysterectomy in Afro-Caribbeans***
  Narmada Lavu, Deepthi Lavu, Hardeep Uppal, Suresh Chandran, Rahul Potluri
  RCOG World congress, Birmingham (U.K), 2016.

- ***Pruritus and Rash –How common are they in Obstetrics and Gynaecology?***

Aishwarya Katta, Metta Arun Kumar, Narmada Lavu, Siri Potluri, Deepthi Lavu, Hardeep Uppal, Suresh Chandran, Rahul Potluri.
RCOG World congress, Birmingham (U.K), 2016.

- ***Extremes of BMI adversely impact pregnancy rates in IVF patients***
  Deepthi Lavu, T. Rani Samyuktha, Kandregula Vijayalakshmi, Macha Lokeshwari, Narakula Sridevi, Narmada Lavu, Pendem Deepika, Mini Mathew, Anuradha Katragadda.
  RCOG World congress, Birmingham (U.K), 2016.

- ***Trends of childbirth in India and UK: A comparative study***.
  Lavu Narmada, Lavu Deepthi, Vijaya M Revankar, Aditi Sunil Shetty, Hardeep Uppal, Suresh Chandran, Rahul Potluri
  RCOG World Congress, Hyderabad (India), 2014.

COMMITTEE RESPONSIBILITIES:

- July 2018-Present GSBS Representative,
  Budget Committee, SGA
  UTMB (Galveston, Texas)

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

- September 2017 – Present Cell biology Journal Club
  The University of Texas Medical Branch at Galveston

- March 2012- Present Indian Medical Association, India.

AWARDS AND HONORS:

- Zelda Zinn Casper Scholarship (UTMB, December 2018).

- 1st place for best Elevator Speech-Responsible conduct for Biomedical research course (BBSC) (UTMB, July 2018).

- 2nd place for best basic science research poster-The Center for Interdisciplinary Research in Women's Health: Poster Session for women's health research (UTMB, May 2018).

- Satish Srivastava Endowed Scholarship (UTMB, December 2017).

- 1st runner up: Intra-departmental quiz competition in reproductive endocrinology (Kasturba Medical College, Mangaluru, Manipal University 2012).

- Medical School (Deccan college of Medical Sciences, August 2006-March 2012)

  - **Gold Medal** (secured first position):
    Anatomy, Physiology, Pharmacology, General Surgery, Pediatrics.

  - Passed with **distinction**:
    Biochemistry, Pathology, Microbiology, Otorhinolaryngology, Ophthalmology.

  - Passed with **honors**:
    Forensic Medicine, Social and Preventive Medicine, Obstetrics and Gynecology, General Medicine.

  - Secured highest overall percentage in class in Medical school.

- 2nd runner up: Inter-College Medical quiz- subject: Forensic Medicine (Mediciti Medical College, Hyderabad, March 2010).

- State Merit Scholarship for the year 2006-07 (Government of Andhra Pradesh, India) based on excellent academic performance at the Intermediate Level Board Examination (12th grade)

- Highest overall percentage in ICSE (Indian Certificate of Secondary Education) Board Examination in 10th grade (Nasr School, Hyderabad, April 2004).

VOLUNTEER SERVICES

- WHO Intensified Pulse Polio Immunization Programme in Hyderabad District, India: Medical Camps held biannually-2008,2009,2010.

- Junior Red Cross Society:2003-2004.

ADDITIONAL TRAINING:

- Rigor and Reproducibility Workshop: Organized by Gulf Coast Consortia, Houston, Texas. October 2018

- ASSET (Advanced Skill Set Embryology Training):
  Department of clinical embryology, Manipal University, Manipal, India.
  December 2015-March 2016.

- Online Certification course on IVF and Embryology: Global Fertility Academy.
  May 2016.

- Preventive Gynecologic oncology: Colposcopy and LEEP Workshop:
  Kasturba Medical College, Mangaluru, Manipal University, India. August 2014.
  Part of the organizing team and participant

- Basic surgical Skills course-Royal College of Obstetrics and Gynecology:
  SDM Medical College, Dharwad, Karnataka, India. December 2013

References

[1] H. Blencowe, S. Cousens, D. Chou, M. Oestergaard, L. Say, A.-B. Moller, M. Kinney, J. Lawn, Born Too Soon: The global epidemiology of 15 million preterm births, Reproductive Health 10(Suppl 1) (2013) S2-S2.
[2] L. Liu, H.L. Johnson, S. Cousens, J. Perin, S. Scott, J.E. Lawn, I. Rudan, H. Campbell, R. Cibulskis, M. Li, C. Mathers, R.E. Black, Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000, Lancet 379(9832) (2012) 2151-61.
[3] S.E. Purisch, C. Gyamfi-Bannerman, Epidemiology of preterm birth, Semin Perinatol 41(7) (2017) 387-391.
[4] R. Romero, S.K. Dey, S.J. Fisher, Preterm labor: one syndrome, many causes, Science 345(6198) (2014) 760-5.
[5] M.K. Mwaniki, M. Atieno, J.E. Lawn, C.R. Newton, Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review, Lancet 379(9814) (2012) 445-52.
[6] K. Ravanos, T. Dagklis, S. Petousis, C. Margioula-Siarkou, Y. Prapas, N. Prapas, Factors implicated in the initiation of human parturition in term and preterm labor: a review, Gynecol Endocrinol 31(9) (2015) 679-83.
[7] R. Menon, I. Boldogh, H.K. Hawkins, M. Woodson, J. Polettini, T.A. Syed, S.J. Fortunato, G.R. Saade, J. Papaconstantinou, R.N. Taylor, Histological evidence of oxidative stress and premature senescence in preterm premature rupture of the human fetal membranes recapitulated in vitro, Am. J. Pathol. 184(6) (2014) 1740-51.
[8] R. Menon, Spontaneous preterm birth, a clinical dilemma: etiologic, pathophysiologic and genetic heterogeneities and racial disparity, Acta Obstet Gynecol Scand 87(6) (2008) 590-600.
[9] R.M. Kamel, The onset of human parturition, Arch Gynecol Obstet 281(6) (2010) 975-82.
[10] S. Mesiano, E.C. Chan, J.T. Fitter, K. Kwek, G. Yeo, R. Smith, Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium, J Clin Endocrinol Metab 87(6) (2002) 2924-30.
[11] D. Pieber, V.C. Allport, F. Hills, M. Johnson, P.R. Bennett, Interactions between progesterone receptor isoforms in myometrial cells in human labour, Mol Hum Reprod 7(9) (2001) 875-9.
[12] R. Romero, H. Munoz, R. Gomez, M. Parra, M. Polanco, V. Valverde, J. Hasbun, J. Garrido, F. Ghezzi, M. Mazor, J.E. Tolosa, M.D. Mitchell, Increase in prostaglandin bioavailability precedes the onset of human parturition, Prostaglandins Leukot Essent Fatty Acids 54(3) (1996) 187-91.
[13] V.C. Allport, D. Pieber, D.M. Slater, R. Newton, J.O. White, P.R. Bennett, Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal', Mol Hum Reprod 7(6) (2001) 581-6.
[14] A.M. Blanks, S. Thornton, The role of oxytocin in parturition, Bjog 110 Suppl 20 (2003) 46-51.

[15] D.C. Wathes, S.C. Borwick, P.M. Timmons, S.T. Leung, S. Thornton, Oxytocin receptor expression in human term and preterm gestational tissues prior to and following the onset of labour, J Endocrinol 161(1) (1999) 143-51.
[16] A. Zicari, C. Ticconi, M. Realacci, O. Cela, C. Santangelo, A. Pietropolli, M.A. Russo, E. Piccione, Hormonal regulation of cytokine release by human fetal membranes at term gestation: effects of oxytocin, hydrocortisone and progesterone on tumour necrosis factor-alpha and transforming growth factor-beta 1 output, J Reprod Immunol 56(1-2) (2002) 123-36.
[17] C. Gustafsson, P. Hummerdal, L. Matthiesen, G. Berg, C. Ekerfelt, J. Ernerudh, Cytokine secretion in decidual mononuclear cells from term human pregnancy with or without labour: ELISPOT detection of IFN-gamma, IL-4, IL-10, TGF-beta and TNF-alpha, J Reprod Immunol 71(1) (2006) 41-56.
[18] T.M. Lindstrom, P.R. Bennett, The role of nuclear factor kappa B in human labour, Reproduction 130(5) (2005) 569-81.
[19] G.D. Bryant-Greenwood, The extracellular matrix of the human fetal membranes: structure and function, Placenta 19(1) (1998) 1-11.
[20] L. Richardson, L. Martin, R. Menon, Characteristics, properties and functionality of fetal membranes: an overlooked area in the field of parturition, Encyclopedia of Reproduction, Elsevier, Amsterdam, the Netherlands, 2018, pp. 387-398.
[21] J.F. Strauss, 3rd, Extracellular matrix dynamics and fetal membrane rupture, Reprod Sci 20(2) (2013) 140-53.
[22] S. Parry, J.F. Strauss, 3rd, Premature rupture of the fetal membranes, N Engl J Med 338(10) (1998) 663-70.
[23] R. Menon, E.A. Bonney, J. Condon, S. Mesiano, R.N. Taylor, Novel concepts on pregnancy clocks and alarms: redundancy and synergy in human parturition, Hum Reprod Update 22(5) (2016) 535-60.
[24] F. Behnia, B.D. Taylor, M. Woodson, M. Kacerovsky, H. Hawkins, S.J. Fortunato, G.R. Saade, R. Menon, Chorioamniotic membrane senescence: a signal for parturition?, Am J Obstet Gynecol 213(3) (2015) 359.e1-16.
[25] R. Menon, Oxidative stress damage as a detrimental factor in preterm birth pathology, Front. Immunol. 5 (2014) 567.
[26] R. Menon, I. Boldogh, R. Urrabaz-Garza, J. Polettini, T.A. Syed, G.R. Saade, J. Papaconstantinou, R.N. Taylor, Senescence of primary amniotic cells via oxidative DNA damage, PLoS One 8(12) (2013) e83416.
[27] T. Force, J.R. Woodgett, Unique and overlapping functions of GSK-3 isoforms in cell differentiation and proliferation and cardiovascular development, J. Biol. Chem. 284(15) (2009) 9643-7.
[28] H. Lee, J.Y. Ro, Differential expression of GSK3beta and pS9GSK3beta in normal human tissues: can pS9GSK3beta be an epithelial marker?, Int. J. Clin. Exp. Pathol. 8(4) (2015) 4064-73.
[29] N. Lavu, L. Richardson, E. Bonney, R. Menon, Glycogen synthase kinase (GSK) 3 in pregnancy and parturition: a systematic review of literature, J Matern Fetal Neonatal Med (2018) 1-221.
[30] E. Beurel, S.F. Grieco, R.S. Jope, Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases, Pharmacol Ther 148 (2015) 114-31.

[31] V. Rider, K. Isuzugawa, M. Twarog, S. Jones, B. Cameron, K. Imakawa, J. Fang, Progesterone initiates Wnt-beta-catenin signaling but estradiol is required for nuclear activation and synchronous proliferation of rat uterine stromal cells, (2006).
[32] L. Lassance, H. Miedl, M. Absenger, F. Diaz-Perez, U. Lang, G. Desoye, U. Hiden, Hyperinsulinemia stimulates angiogenesis of human fetoplacental endothelial cells: a possible role of insulin in placental hypervascularization in diabetes mellitus, Journal of Clinical Endocrinology & Metabolism 98(9) (2013) E1438-47.
[33] T.M. Thornton, G. Pedraza-Alva, B. Deng, C.D. Wood, A. Aronshtam, J.L. Clements, G. Sabio, R.J. Davis, D.E. Matthews, B. Doble, M. Rincon, Phosphorylation by p38 MAPK as an Alternative Pathway for GSK3β Inactivation, Science (New York, N.Y.) 320(5876) (2008) 667-670.
[34] J. Lee, S.K. Banu, J.A. McCracken, J.A. Arosh, Early pregnancy modulates survival and apoptosis pathways in the corpus luteum in sheep, Reproduction (Cambridge, England) 151(3) (2016) 187-202.
[35] S. Bredeson, J. Papaconstantinou, J.H. Deford, T. Kechichian, T.A. Syed, G.R. Saade, R. Menon, HMGB1 promotes a p38MAPK associated non-infectious inflammatory response pathway in human fetal membranes, PLoS One 9(12) (2014) e113799.
[36] J. Liu, F. Gao, Y.F. Liu, H.T. Dou, J.Q. Yan, Z.M. Fan, Z.M. Yang, HB-EGF regulates Prss56 expression during mouse decidualization via EGFR/ERK/EGR2 signaling pathway, J. Endocrinol. 234(3) (2017) 247-254.
[37] J. Zmijanac Partl, V. Karin, A. Skrtic, T. Nikuseva-Martic, A. Serman, J. Mlinarec, M. Curkovic-Perica, S. Vranic, L. Serman, Negative regulators of Wnt signaling pathway SFRP1 and SFRP3 expression in preterm and term pathologic placentas, J. Matern. Fetal Neonatal Med. (2017) 1-9.
[38] C. Sutherland, What Are the bona fide GSK3 Substrates?, Int. J. Alzheimers Dis. 2011 (2011) 505607.
[39] D. Voskas, L.S. Ling, J.R. Woodgett, Does GSK-3 provide a shortcut for PI3K activation of Wnt signalling?, F1000 Biol. Rep. 2 (2010) 82.
[40] T. Valenta, G. Hausmann, K. Basler, The many faces and functions of β-catenin, The EMBO journal 31(12) (2012) 2714-2736.
[41] P.D. Ray, B.-W. Huang, Y. Tsuji, Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling, Cellular signalling 24(5) (2012) 981-990.
[42] Q. Ma, Role of nrf2 in oxidative stress and toxicity, Annual review of pharmacology and toxicology 53 (2013) 401-426.
[43] T. Nguyen, P. Nioi, C.B. Pickett, The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress, The Journal of biological chemistry 284(20) (2009) 13291-13295.
[44] C.A. Silva-Islas, P.D. Maldonado, Canonical and non-canonical mechanisms of Nrf2 activation, Pharmacological Research 134 (2018) 92-99.
[45] H.K. Bryan, A. Olayanju, C.E. Goldring, B.K. Park, The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation, Biochemical Pharmacology 85(6) (2013) 705-717.
[46] A. Loboda, M. Damulewicz, E. Pyza, A. Jozkowicz, J. Dulak, Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily

conserved mechanism, Cellular and molecular life sciences : CMLS 73(17) (2016) 3221-3247.
[47] A. Cuadrado, S. Kügler, I. Lastres-Becker, Pharmacological targeting of GSK-3 and NRF2 provides neuroprotection in a preclinical model of tauopathy, Redox biology 14 (2017) 522-534.
[48] M. Culbreth, M. Aschner, GSK-3β, a double-edged sword in Nrf2 regulation: Implications for neurological dysfunction and disease, F1000Research 7 (2018) 1043-1043.
[49] M. Salazar, A.I. Rojo, D. Velasco, R.M. de Sagarra, A. Cuadrado, Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2, J Biol Chem 281(21) (2006) 14841-51.
[50] Y. Jiang, H. Bao, Y. Ge, W. Tang, D. Cheng, K. Luo, G. Gong, R. Gong, Therapeutic targeting of GSK3β enhances the Nrf2 antioxidant response and confers hepatic cytoprotection in hepatitis C, Gut 64(1) (2015) 168-179.
[51] M. Tkach, C. Thery, Communication by Extracellular Vesicles: Where We Are and Where We Need to Go, Cell 164(6) (2016) 1226-1232.
[52] S. Sheller, J. Papaconstantinou, R. Urrabaz-Garza, L. Richardson, G. Saade, C. Salomon, R. Menon, Amnion-Epithelial-Cell-Derived Exosomes Demonstrate Physiologic State of Cell under Oxidative Stress, PLoS ONE 11(6) (2016) e0157614.
[53] M. H. Rashed, E. Bayraktar, G. K. Helal, M.F. Abd-Ellah, P. Amero, A. Chavez-Reyes, C. Rodriguez-Aguayo, Exosomes: From Garbage Bins to Promising Therapeutic Targets, International Journal of Molecular Sciences 18(3) (2017) 538.
[54] W.S. Toh, R.C. Lai, B. Zhang, S.K. Lim, MSC exosome works through a protein-based mechanism of action, Biochem Soc Trans 46(4) (2018) 843-853.
[55] M.D. Mitchell, H.N. Peiris, M. Kobayashi, Y.Q. Koh, G. Duncombe, S.E. Illanes, G.E. Rice, C. Salomon, Placental exosomes in normal and complicated pregnancy, Am J Obstet Gynecol 213(4 Suppl) (2015) S173-81.
[56] E.E. Hadley, S. Sheller-Miller, G. Saade, C. Salomon, S. Mesiano, R.N. Taylor, B.D. Taylor, R. Menon, Amnion Epithelial Cell Derived Exosomes Induce Inflammatory Changes in Uterine Cells, Am J Obstet Gynecol (2018).
[57] R. Menon, Oxidative Stress Damage as a Detrimental Factor in Preterm Birth Pathology, Frontiers in Immunology 5 (2014) 567.
[58] E.H. Dutta, F. Behnia, I. Boldogh, G.R. Saade, B.D. Taylor, M. Kacerovský, R. Menon, Oxidative stress damage-associated molecular signaling pathways differentiate spontaneous preterm birth and preterm premature rupture of the membranes, MHR: Basic science of reproductive medicine 22(2) (2016) 143-157.
[59] C.L. Dixon, L. Richardson, S. Sheller-Miller, G. Saade, R. Menon, A distinct mechanism of senescence activation in amnion epithelial cells by infection, inflammation, and oxidative stress, Am J Reprod Immunol 79(3) (2018).
[60] J.A. McCubrey, L.S. Steelman, F.E. Bertrand, N.M. Davis, S.L. Abrams, G. Montalto, A.B. D'Assoro, M. Libra, F. Nicoletti, R. Maestro, J. Basecke, L. Cocco, M. Cervello, A.M. Martelli, Multifaceted roles of GSK-3 and Wnt/β-catenin in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention, Leukemia 28 (2013) 15.

[61] H. Eldar-Finkelman, A. Martinez, GSK-3 Inhibitors: Preclinical and Clinical Focus on CNS, Frontiers in molecular neuroscience 4 (2011) 32-32.
[62] R.S. Jope, C.J. Yuskaitis, E. Beurel, Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics, Neurochemical research 32(4-5) (2007) 577-595.
[63] L. Lassance, H. Miedl, M. Absenger, F. Diaz-Perez, U. Lang, G. Desoye, U. Hiden, Hyperinsulinemia stimulates angiogenesis of human fetoplacental endothelial cells: a possible role of insulin in placental hypervascularization in diabetes mellitus, J. Clin. Endocrinol. Metab. 98(9) (2013) E1438-47.
[64] Y. Zhou, M.J. Gormley, N.M. Hunkapiller, M. Kapidzic, Y. Stolyarov, V. Feng, M. Nishida, P.M. Drake, K. Bianco, F. Wang, M.T. McMaster, S.J. Fisher, Reversal of gene dysregulation in cultured cytotrophoblasts reveals possible causes of preeclampsia, J. Clin. Invest. 123(7) (2013) 2862-72.
[65] Y. Hua, S. Ding, H. Cheng, H. Luo, X. Zhu, Tanshinone IIA increases aquaporins expression in human amniotic epithelial WISH cells by stimulating GSK-3beta phosphorylation, Clin. Chim. Acta 473 (2017) 204-212.
[66] J.R. Woodgett, Molecular cloning and expression of glycogen synthase kinase-3/factor A, EMBO J. 9(8) (1990) 2431-8.
[67] K.P. Hoeflich, J. Luo, E.A. Rubie, M.S. Tsao, O. Jin, J.R. Woodgett, Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation, Nature 406(6791) (2000) 86-90.
[68] A. Moustakas, C.H. Heldin, Mechanisms of TGFbeta-Induced Epithelial-Mesenchymal Transition, Journal of clinical medicine 5(7) (2016).
[69] R.K. Bikkavilli, C.C. Malbon, Mitogen-activated protein kinases and Wnt/beta-catenin signaling: Molecular conversations among signaling pathways, Commun. Integr. Biol. 2(1) (2009) 46-9.
[70] J.A. McCubrey, L.S. Steelman, F.E. Bertrand, N.M. Davis, M. Sokolosky, S.L. Abrams, G. Montalto, A.B. D'Assoro, M. Libra, F. Nicoletti, R. Maestro, J. Basecke, D. Rakus, A. Gizak, Z.N. Demidenko, L. Cocco, A.M. Martelli, M. Cervello, GSK-3 as potential target for therapeutic intervention in cancer, Oncotarget 5(10) (2014) 2881-911.
[71] D.F. Stroup, J.A. Berlin, S.C. Morton, I. Olkin, G.D. Williamson, D. Rennie, D. Moher, B.J. Becker, T.A. Sipe, S.B. Thacker, Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group, JAMA 283(15) (2000) 2008-12.
[72] A. Liberati, D.G. Altman, J. Tetzlaff, C. Mulrow, P.C. Gotzsche, J.P. Ioannidis, M. Clarke, P.J. Devereaux, J. Kleijnen, D. Moher, The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration, BMJ 339 (2009) b2700.
[73] E.E. Hadley, L.S. Richardson, M.R. Torloni, R. Menon, Gestational tissue inflammatory biomarkers at term labor: A systematic review of literature, Am. J. Reprod. Immunol. 79(2) (2018).
[74] A. Boronkai, N.G. Than, R. Magenheim, S. Bellyei, A. Szigeti, P. Deres, B. Hargitai, B. Sumegi, Z. Papp, J. Rigo, Jr., Extremely high maternal alkaline phosphatase serum concentration with syncytiotrophoblastic origin, J. Clin. Pathol. 58(1) (2005) 72-6.

[75] B.A. O'Connell, K.M. Moritz, D.W. Walker, H. Dickinson, Treatment of pregnant spiny mice at mid gestation with a synthetic glucocorticoid has sex-dependent effects on placental glycogen stores, Placenta 34(10) (2013) 932-40.
[76] V. Rider, K. Isuzugawa, M. Twarog, S. Jones, B. Cameron, K. Imakawa, J. Fang, Progesterone initiates Wnt-beta-catenin signaling but estradiol is required for nuclear activation and synchronous proliferation of rat uterine stromal cells, J. Endocrinol. 191(3) (2006) 537-48.
[77] M.H. Chiang, F.Y. Liang, C.P. Chen, C.W. Chang, M.L. Cheong, L.J. Wang, C.Y. Liang, F.Y. Lin, C.C. Chou, H. Chen, Mechanism of hypoxia-induced GCM1 degradation: implications for the pathogenesis of preeclampsia, J. Biol. Chem. 284(26) (2009) 17411-9.
[78] R. Lim, M. Lappas, A novel role for GSK3 in the regulation of the processes of human labour, Reproduction 149(2) (2015) 189-202.
[79] Y. Astuti, K. Nakabayashi, M. Deguchi, Y. Ebina, H. Yamada, Human recombinant H2 relaxin induces AKT and GSK3beta phosphorylation and HTR-8/SVneo cell proliferation, Kobe J. Med. Sci. 61(1) (2015) E1-8.
[80] A.K. Roseweir, A.A. Katz, R.P. Millar, Kisspeptin-10 inhibits cell migration in vitro via a receptor-GSK3 beta-FAK feedback loop in HTR8SVneo cells, Placenta 33(5) (2012) 408-15.
[81] I. Fischer, M. Weber, C. Kuhn, J.S. Fitzgerald, S. Schulze, K. Friese, H. Walzel, U.R. Markert, U. Jeschke, Is galectin-1 a trigger for trophoblast cell fusion?: the MAP-kinase pathway and syncytium formation in trophoblast tumour cells BeWo, Mol. Hum. Reprod. 17(12) (2011) 747-57.
[82] M.N. Lague, J. Detmar, M. Paquet, A. Boyer, J.S. Richards, S.L. Adamson, D. Boerboom, Decidual PTEN expression is required for trophoblast invasion in the mouse, Am. J. Physiol. Endocrinol. Metab. 299(6) (2010) E936-46.
[83] H.L. LaMarca, P.R. Dash, K. Vishnuthevan, E. Harvey, D.E. Sullivan, C.A. Morris, G.S. Whitley, Epidermal growth factor-stimulated extravillous cytotrophoblast motility is mediated by the activation of PI3-K, Akt and both p38 and p42/44 mitogen-activated protein kinases, Hum. Reprod. 23(8) (2008) 1733-41.
[84] J. Kim, G. Song, H. Gao, J.L. Farmer, M.C. Satterfield, R.C. Burghardt, G. Wu, G.A. Johnson, T.E. Spencer, F.W. Bazer, Insulin-like growth factor II activates phosphatidylinositol 3-kinase-protooncogenic protein kinase 1 and mitogen-activated protein kinase cell Signaling pathways, and stimulates migration of ovine trophectoderm cells, Endocrinology 149(6) (2008) 3085-94.
[85] S. Sonderegger, P. Haslinger, A. Sabri, C. Leisser, J.V. Otten, C. Fiala, M. Knofler, Wingless (Wnt)-3A induces trophoblast migration and matrix metalloproteinase-2 secretion through canonical Wnt signaling and protein kinase B/AKT activation, Endocrinology 151(1) (2010) 211-20.
[86] H.W. Yung, S. Calabrese, D. Hynx, B.A. Hemmings, I. Cetin, D.S. Charnock-Jones, G.J. Burton, Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction, Am. J. Pathol. 173(2) (2008) 451-62.
[87] P.R. Dash, G.S. Whitley, L.J. Ayling, A.P. Johnstone, J.E. Cartwright, Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin

mediated up-regulation of inducible nitric oxide synthase, Cell. Signal. 17(5) (2005) 571-80.
[88] L. Laviola, S. Perrini, G. Belsanti, A. Natalicchio, C. Montrone, A. Leonardini, A. Vimercati, M. Scioscia, L. Selvaggi, R. Giorgino, P. Greco, F. Giorgino, Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling, Endocrinology 146(3) (2005) 1498-505.
[89] X. Hou, E.W. Arvisais, J.S. Davis, Luteinizing hormone stimulates mammalian target of rapamycin signaling in bovine luteal cells via pathways independent of AKT and mitogen-activated protein kinase: modulation of glycogen synthase kinase 3 and AMP-activated protein kinase, Endocrinology 151(6) (2010) 2846-57.
[90] C.F. Yen, S.H. Kim, S.K. Liao, C. Atabekoglu, S. Uckac, A. Arici, S. Arlier, C.L. Lee, H.S. Wang, U.A. Kayisli, Increased expression of integrin-linked kinase during decidualization regulates the morphological transformation of endometrial stromal cells, Fertil. Steril. 107(3) (2017) 803-812.
[91] S.J. Fisher, Why is placentation abnormal in preeclampsia?, Am. J. Obstet. Gynecol. 213(4 Suppl) (2015) S115-22.
[92] H.W. Yung, F. Colleoni, D. Atkinson, E. Cook, A.J. Murray, G.J. Burton, D.S. Charnock-Jones, Influence of speed of sample processing on placental energetics and signalling pathways: implications for tissue collection, Placenta 35(2) (2014) 103-8.
[93] B. Feng, L. Qiu, C. Ye, L. Chen, Y. Fu, W. Sun, Exposure to a 50-Hz magnetic field induced mitochondrial permeability transition through the ROS/GSK-3beta signaling pathway, Int. J. Radiat. Biol. 92(3) (2016) 148-55.
[94] X. Cheng, S.J. Chapple, B. Patel, W. Puszyk, D. Sugden, X. Yin, M. Mayr, R.C. Siow, G.E. Mann, Gestational diabetes mellitus impairs Nrf2-mediated adaptive antioxidant defenses and redox signaling in fetal endothelial cells in utero, Diabetes 62(12) (2013) 4088-97.
[95] S.A. Bramer, A. Macedo, C. Klein, Hexokinase 2 drives glycogen accumulation in equine endometrium at day 12 of diestrus and pregnancy, Reprod. Biol. Endocrinol. 15(1) (2017) 4.
[96] L. Ruel, M. Bourouis, P. Heitzler, V. Pantesco, P. Simpson, Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch, Nature 362(6420) (1993) 557-60.
[97] E. Siegfried, T.B. Chou, N. Perrimon, wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate, Cell 71(7) (1992) 1167-79.
[98] X.Y. Hou, E.W. Arvisais, J.S. Davis, Luteinizing Hormone Stimulates Mammalian Target of Rapamycin Signaling in Bovine Luteal Cells via Pathways Independent of AKT and Mitogen-Activated Protein Kinase: Modulation of Glycogen Synthase Kinase 3 and AMP-Activated Protein Kinase, Endocrinology 151(6) (2010) 2846-2857.
[99] V. Rider, K. Isuzugawa, M. Twarog, S. Jones, B. Cameron, K. Imakawa, J.W. Fang, Progesterone initiates Wnt-beta-catenin signaling but estradiol is required for nuclear activation and synchronous proliferation of rat uterine stromal cells, Journal of Endocrinology 191(3) (2006) 537-548.
[100] B.A. O'Connell, K.M. Moritz, D.W. Walker, H. Dickinson, Treatment of pregnant spiny mice at mid gestation with a synthetic glucocorticoid has sex-dependent effects on placental glycogen stores, Placenta 34(10) (2013) 932-940.

[101] E. Rodriguez-Carballo, B. Gamez, F. Ventura, p38 MAPK Signaling in Osteoblast Differentiation, Front Cell Dev Biol 4 (2016) 40.
[102] N. Lavu, L. Richardson, E. Radnaa, T. Kechichian, R. Urrabaz-Garza, S. Sheller-Miller, E. Bonney, R. Menon, Oxidative Stress Induced Downregulation of Glycogen Synthase kinase 3β (GSK3β) in Fetal Membranes Promotes Cellular Senescence, Biology of Reproduction (in press), 2019.
[103] L. Martin, L. Richardson, R. Menon, Characteristics, properties and functionality of fetal membranes: an overlooked area in the field of parturition, Encyclopedia of Reproduction, Elsevier, Amsterdam, the Netherlands, 2018, pp. 387-398.
[104] L. Richardson, C.L. Dixon, L. Aguilera-Aguirre, R. Menon, Oxidative stress-induced TGF-beta/TAB1-mediated p38MAPK activation in human amnion epithelial cells, Biol Reprod 99(5) (2018) 1100-1112.
[105] T.M. Thornton, G. Pedraza-Alva, B. Deng, C.D. Wood, A. Aronshtam, J.L. Clements, G. Sabio, R.J. Davis, D.E. Matthews, B. Doble, M. Rincon, Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation, Science 320(5876) (2008) 667-70.
[106] B. Doble, J.R. Woodgett, GSK-3: tricks of the trade for a multi-tasking kinase, Journal of cell science 116(Pt 7) (2003) 1175-1186.
[107] C. Sutherland, What Are the bona fide GSK3 Substrates?, International Journal of Alzheimer's Disease 2011 (2011) 505607.
[108] X. Cheng, S.J. Chapple, B. Patel, W. Puszyk, D. Sugden, X. Yin, M. Mayr, R.C. Siow, G.E. Mann, Gestational diabetes mellitus impairs Nrf2-mediated adaptive antioxidant defenses and redox signaling in fetal endothelial cells in utero, (2013).
[109] L.S. Richardson, G. Vargas, T. Brown, L. Ochoa, S. Sheller-Miller, G.R. Saade, R.N. Taylor, R. Menon, Discovery and Characterization of Human Amniochorionic Membrane Microfractures, Am J Pathol 187(12) (2017) 2821-2830.
[110] J. Jin, L. Richardson, S. Sheller-Miller, N. Zhong, R. Menon, Oxidative stress induces p38MAPK-dependent senescence in the feto-maternal interface cells, Placenta 67 (2018) 15-23.
[111] M.L. Casey, P.C. MacDonald, Interstitial collagen synthesis and processing in human amnion: a property of the mesenchymal cells, Biol Reprod 55(6) (1996) 1253-60.
[112] B.L. Sato, E.S. Collier, S.A. Vermudez, A.D. Junker, C.E. Kendal-Wright, Human amnion mesenchymal cells are pro-inflammatory when activated by the Toll-like receptor 2/6 ligand, macrophage-activating lipoprotein-2, Placenta 44 (2016) 69-79.
[113] K. Balamurugan, S. Sharan, K.D. Klarmann, Y. Zhang, V. Coppola, G.H. Summers, T. Roger, D.K. Morrison, J.R. Keller, E. Sterneck, FBXW7α attenuates inflammatory signalling by downregulating C/EBPδ and its target gene Tlr4, Nature Communications 4 (2013) 1662.
[114] S. Sheller-Miller, R. Urrabaz-Garza, G. Saade, R. Menon, Damage-Associated molecular pattern markers HMGB1 and cell-Free fetal telomere fragments in oxidative-Stressed amnion epithelial cell-Derived exosomes, J Reprod Immunol 123 (2017) 3-11.
[115] J. Polettini, L.S. Richardson, R. Menon, Oxidative stress induces senescence and sterile inflammation in murine amniotic cavity, Placenta 63 (2018) 26-31.
[116] L.F. Martin, L.S. Richardson, M.G. da Silva, S. Sheller-Miller, R. Menon, Dexamethasone Induces Primary Amnion Epithelial Cell Senescence through Telomere-P21 Associated Pathway, Biol Reprod (2019).

[117] L. Richardson, R. Menon, Proliferative, Migratory, and Transition Properties Reveal Metastate of Human Amnion Cells, Am J Pathol 188(9) (2018) 2004-2015.
[118] L.G. Han, Q.-L. Zhao, T. Yoshida, M. Okabe, C. Soko, M.U. Rehman, T. Kondo, T. Nikaido, Differential response of immortalized human amnion mesenchymal and epithelial cells against oxidative stress, Free Radical Biology and Medicine 135 (2019) 79-86.
[119] T. Quast, F. Eppler, V. Semmling, C. Schild, Y. Homsi, S. Levy, T. Lang, C. Kurts, W. Kolanus, CD81 is essential for the formation of membrane protrusions and regulates Rac1-activation in adhesion-dependent immune cell migration, Blood 118(7) (2011) 1818-27.
[120] Y. Astuti, K. Nakabayashi, M. Deguchi, Y. Ebina, H. Yamada, Human recombinant H2 relaxin induces AKT and GSK3beta phosphorylation and HTR-8/SVneo cell proliferation, Kobe Journal of Medical Sciences 61(1) (2015) E1-8.
[121] S. Sonderegger, P. Haslinger, A. Sabri, C. Leisser, J.V. Otten, C. Fiala, M. Knofler, Wingless (Wnt)-3A Induces Trophoblast Migration and Matrix Metalloproteinase-2 Secretion through Canonical Wnt Signaling and Protein Kinase B/AKT Activation, Endocrinology 151(1) (2010) 211-220.
[122] I. Fischer, M. Weber, C. Kuhn, J.S. Fitzgerald, S. Schulze, K. Friese, H. Walzel, U.R. Markert, U. Jeschke, Is galectin-1 a trigger for trophoblast cell fusion?: the MAP-kinase pathway and syncytium formation in trophoblast tumour cells BeWo, Molecular Human Reproduction 17(12) (2011) 747-757.
[123] R. Mancinelli, G. Carpino, S. Petrungaro, C.L. Mammola, L. Tomaipitinca, A. Filippini, A. Facchiano, E. Ziparo, C. Giampietri, Multifaceted Roles of GSK-3 in Cancer and Autophagy-Related Diseases, Oxid Med Cell Longev 2017 (2017) 4629495.
[124] Y.M. Kim, Y.H. Seo, C.B. Park, S.H. Yoon, G. Yoon, Roles of GSK3 in metabolic shift toward abnormal anabolism in cell senescence, Ann N Y Acad Sci 1201 (2010) 65-71.
[125] Y.H. Seo, H.J. Jung, H.T. Shin, Y.M. Kim, H. Yim, H.Y. Chung, I.K. Lim, G. Yoon, Enhanced glycogenesis is involved in cellular senescence via GSK3/GS modulation, Aging Cell 7(6) (2008) 894-907.
[126] Y.-M. Kim, I. Song, Y.-H. Seo, G. Yoon, Glycogen Synthase Kinase 3 Inactivation Induces Cell Senescence through Sterol Regulatory Element Binding Protein 1-Mediated Lipogenesis in Chang Cells, Endocrinology and metabolism (Seoul, Korea) 28(4) (2013) 297-308.
[127] R. Menon, J. Papaconstantinou, p38 Mitogen activated protein kinase (MAPK): a new therapeutic target for reducing the risk of adverse pregnancy outcomes, Expert Opin Ther Targets 20(12) (2016) 1397-1412.
[128] G.V. Vijay, N. Zhao, P. Den Hollander, M.J. Toneff, R. Joseph, M. Pietila, J.H. Taube, T.R. Sarkar, E. Ramirez-Pena, S.J. Werden, M. Shariati, R. Gao, M. Sobieski, C.C. Stephan, N. Sphyris, N. Miura, P. Davies, J.T. Chang, R. Soundararajan, J.M. Rosen, S.A. Mani, GSK3β regulates epithelial-mesenchymal transition and cancer stem cell properties in triple-negative breast cancer, Breast Cancer Research 21(1) (2019) 37.
[129] J. Luo, The role of GSK3beta in the development of the central nervous system, Frontiers in biology 7(3) (2012) 212-220.

[130] P. Patel, J.R. Woodgett, Chapter Eight - Glycogen Synthase Kinase 3: A Kinase for All Pathways?, in: A. Jenny (Ed.), Current Topics in Developmental Biology, Academic Press2017, pp. 277-302.
[131] R. Anton, H.A. Kestler, M. Kuhl, Beta-catenin signaling contributes to stemness and regulates early differentiation in murine embryonic stem cells, FEBS Lett 581(27) (2007) 5247-54.
[132] V.C. Pai, C.-C. Hsu, T.-S. Chan, W.-Y. Liao, C.-P. Chuu, W.-Y. Chen, C.-R. Li, C.-Y. Lin, S.-P. Huang, L.-T. Chen, K.K. Tsai, Correction: ASPM promotes prostate cancer stemness and progression by augmenting Wnt−Dvl-3−β-catenin signaling, Oncogene 38(8) (2019) 1354-1354.
[133] S. Shang, F. Hua, Z.-W. Hu, The regulation of β-catenin activity and function in cancer: therapeutic opportunities, Oncotarget 8(20) (2017) 33972-33989.
[134] L. Larue, F. Luciani, M. Kumasaka, D. Champeval, N. Demirkan, J. Bonaventure, V. Delmas, Bypassing melanocyte senescence by β-catenin: A novel way to promote melanoma, Pathologie Biologie 57(7) (2009) 543-547.
[135] L.M. Tian, H.F. Xie, X. Xiao, T. Yang, Y.H. Hu, W.Z. Wang, L.S. Liu, X. Chen, J. Li, Study on the roles of beta-catenin in hydrogen peroxide-induced senescence in human skin fibroblasts, Exp Dermatol 20(10) (2011) 836-8.
[136] K. Hoffmeyer, A. Raggioli, S. Rudloff, R. Anton, A. Hierholzer, I. Del Valle, K. Hein, R. Vogt, R. Kemler, Wnt/beta-catenin signaling regulates telomerase in stem cells and cancer cells, Science 336(6088) (2012) 1549-54.
[137] F. Behnia, S. Sheller, R. Menon, Mechanistic Differences Leading to Infectious and Sterile Inflammation, Am J Reprod Immunol 75(5) (2016) 505-18.
[138] R. Lim, G. Barker, M. Lappas, The transcription factor Nrf2 is decreased after spontaneous term labour in human fetal membranes where it exerts anti-inflammatory properties, Placenta 36(1) (2015) 7-17.
[139] P. Hiebert, M.S. Wietecha, M. Cangkrama, E. Haertel, E. Mavrogonatou, M. Stumpe, H. Steenbock, S. Grossi, H.D. Beer, P. Angel, J. Brinckmann, D. Kletsas, J. Dengjel, S. Werner, Nrf2-Mediated Fibroblast Reprogramming Drives Cellular Senescence by Targeting the Matrisome, Dev Cell 46(2) (2018) 145-161.e10.
[140] S. Polvani, M. Tarocchi, A. Galli, PPARγ and Oxidative Stress: Con(β) Catenating NRF2 and FOXO, PPAR research 2012 (2012) 641087-641087.
[141] A. Vallée, Y. Lecarpentier, Crosstalk Between Peroxisome Proliferator-Activated Receptor Gamma and the Canonical WNT/β-Catenin Pathway in Chronic Inflammation and Oxidative Stress During Carcinogenesis, Frontiers in immunology 9 (2018) 745-745.
[142] Y. Lecarpentier, A. Vallée, Opposite Interplay between PPAR Gamma and Canonical Wnt/Beta-Catenin Pathway in Amyotrophic Lateral Sclerosis, Frontiers in neurology 7 (2016) 100-100.
[143] Y. Lecarpentier, V. Claes, A. Vallée, J.-L. Hébert, Interactions between PPAR Gamma and the Canonical Wnt/Beta-Catenin Pathway in Type 2 Diabetes and Colon Cancer, PPAR research 2017 (2017) 5879090-5879090.
[144] G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla, A. Bitto, Oxidative Stress: Harms and Benefits for Human Health, Oxidative medicine and cellular longevity 2017 (2017) 8416763-8416763.
[145] J. Diaz-Castro, J. Florido, N. Kajarabille, S. Prados, C. de Paco, O. Ocon, M. Pulido-Moran, J.J. Ochoa, A new approach to oxidative stress and inflammatory

signaling during labour in healthy mothers and neonates, Oxid Med Cell Longev 2015 (2015) 178536.
[146] J. Polettini, F. Behnia, B.D. Taylor, G.R. Saade, R.N. Taylor, R. Menon, Telomere Fragment Induced Amnion Cell Senescence: A Contributor to Parturition?, PLoS One 10(9) (2015) e0137188.