The Discovery of a Regulatory Role of Prohibitin-1 in Testosterone Production and the Intracellular Cholesterol Pool in Basal Steroidogenesis

by

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CONTRIBUTIONS OF AUTHORS

This modified sandwich thesis comprises of three multi-authored manuscripts: one review and two research articles published in peer-reviewed scientific journals. I contributed to the majority of the work embodied in this thesis and have been credited with the first authorship in all three manuscripts. The manuscript wise author contribution details are as follows:

1. **The expanding role of mitochondria, autophagy and lipophagy in steroidogenesis.**
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   Geetika is the first author and was responsible for conceptualization, literature review, writing original draft preparation, review and editing along with Simarjit Kaur Sidhu and Suresh Mishra.

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   Geetika is the first author and along with Suresh Mishra was responsible for conceptualizing study, designing, performing experiments and data analyses. Simarjit Kaur Sidhu performed Y-1 cells western blots.

THESIS ABSTRACT

The fundamental framework of steroidogenesis across steroidogenic cells is similar, especially the initial mitochondrial steps involving cholesterol translocation across outer mitochondrial membrane (OMM) by the START domain containing proteins (e.g., StAR) and subsequent conversion to pregnenolone by the enzyme P450scc in the inner mitochondrial membrane (IMM). Thus, cholesterol and mitochondria are essential and highly interconnected in steroidogenesis. However, our understanding of this conserved process in steroidogenesis remains limited. Particularly, the transport of cholesterol from the OMM to the IMM. Moreover, growing evidence suggest an important role of autophagy/lipophagy and mitochondrial dynamics in regulating steroidogenic cholesterol homeostasis. However, a potential role the intracellular cholesterol pool in itself in the regulation steroidogenic events remains unexplored. I investigated an unexpected testicular phenotype of two transgenic mouse models and found that the male transgenic mice that expressed a mutant form of a mitochondrial protein prohibitin-1 (PHB1Tyr^{114}Phe) from the *Fabp-4* gene promoter displayed smaller testes, higher testosterone levels and lower gonadotropin levels as compared with age matched PHB-1 overexpressing and wild-type mice. Additionally, I found StAR and P450scc as interacting partners of PHB1 in Leydig cell mitochondria. Further analysis of testis and Leydig cells from the transgenic mice revealed that PHB1 plays a regulatory role in coordinating cell signaling, cholesterol homeostasis, and mitochondrial biology pertaining to steroidogenesis. Moreover, I tested the hypothesis that intracellular cholesterol pool in steroidogenic cells plays a role in regulating cell-intrinsic factors and events pertaining steroidogenesis. I found that the depletion of intracellular cholesterol pool in steroidogenic cells (i.e., MA-10, Y-1 and BeWo cells) induces autophagy, affects mitochondrial dynamics, and upregulates steroidogenic factors and basal steroidogenesis. Interestingly, cholesterol depletion-induced changes in steroidogenic cells were found to occur independent of hormone stimulation suggesting a role of cholesterol in basal steroidogenesis. In conclusion, my findings provide novel insights into the role of PHB1 in coordinating steroidogenesis in Leydig cell and a role of the intracellular cholesterol pool in basal steroidogenesis. The implications of my findings are broad as cholesterol is the common substrate for all steroid hormones and the initial stages of steroidogenesis are indistinguishable across steroidogenic cells.

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DEDICATION

Every challenging work needs self-efforts as well as guidance and support from others to move in the right direction.

This thesis is dedicated:

To my mother and best friend: **Mrs. Saroj Bassi and Vijay K Nahar**

For constantly reminding me “where there is a will, there is a way” and motivating and inspiring me to achieve my goals. Thank you from the bottom of my heart for your unconditional love and support. Today wherever I am, it is all because of the trust you both have in me and the unconditional love that you have provided in these years. I cannot thank enough! I love you both the most!

TABLE OF CONTENTS

COVER i
CONTRIBUTIONS OF AUTHORS ii
THESIS ABSTRACT iii
ACKNOWLEDGEMENTS iv
DEDICATION vi
TABLE OF CONTENTS vii
LIST OF FIGURES xiii
LIST OF TABLES xv
LIST OF OPEN ACCESS AND COPYRIGHT MATERIALS xvi
LIST OF ABBREVIATIONS xvii

CHAPTER 1

INTRODUCTION

The Expanding Role of Mitochondria, Autophagy and Lipophagy in Steroidogenesis 1

1.0 Abstract 2

1.1 Introduction 3

1.2 Steroidogenic cells and steroidogenic mitochondria 6

1.2.1 Steroidogenesis and mitochondrial structures—the role of steroidogenic enzymes 8

1.2.2 Hormone-induced and cell-intrinsic processes in steroidogenic cells 9

1.2.3 The importance of mitochondrial dynamics in steroidogenesis 10

1.2.4 Steroidogenic mitochondria- a comparative account 11

1.3 Placental steroidogenesis- what we can learn from the similarities and differences with adrenal and gonadal steroidogenesis? 14

1.4 Cholesterol- its importance as a starting substrate and need for cholesterol import to mitochondria 16

1.4.1 Heterogeneity in cholesterol distribution and cellular compartmentalization of steroidogenesis 18

1.5 Autophagy and lipophagy in cholesterol homeostasis and steroidogenesis 20

1.5.1 Autophagy in steroidogenesis- A conserved mechanism? 24

1.6 Prohibitin- a putative novel player in the steroidogenic mitochondria and cells at large 25

1.6.1 The relationship between PHB family proteins and cholesterol 26

1.7 Outstanding questions and future research directions 28

Appendix A. Outstanding questions 28

CHAPTER 2

STUDY RATIONALE, HYPOTHESIS AND OBJECTIVES.. 30

2.1 Study rationale 30

2.2 Hypothesis 31

2.3 Objectives 31

2.3.1 Characterize testicular phenotype in PHB1 transgenic mice and explore the role of PHB1 in Leydig cell biology pertaining steroidogenesis 31

2.3.2 Identify PHB's interacting partners in Leydig cell mitochondria 31

2.3.3 Examine the role of PHB1 in cell signaling pathways in Leydig cells 31

2.3.4 Investigate the role of intracellular cholesterol pool in steroidogenic cells 31

CHAPTER 3

MATERIALS AND METHODS 33

3.1 Materials

3.1.1 Chemicals and reagents 33

3.1.2 Media for bacterial culture 36

3.1.3 Buffers 36

3.2 Methods

3.2.1 Animal models… 36

3.2.2 Testis retrieval 37

3.2.3 Primary Leydig cell (LC) isolation and culture 37

3.2.4 Histological analysis 37

3.2.5 Transmission electron microscopy (TEM) 38

3.2.6 Plasmid DNA transformation of competent *E. coli* cells 38

3.2.7 Plasmid DNA isolation 38

3.2.8 Cell culture 39

3.2.9 Cell treatment 39

3.2.10 Depletion of intracellular cholesterol pool in steroidogenic cells 39

3.2.11 Cell transfections 40

3.2.12 CRISPR/Cas9-Phb-MA-10 cells 40

3.2.13 MTT reduction assay 41

3.2.14 Western blotting 41

3.2.15 Immunoprecipitation 42

3.2.16 Cholesterol binding assay 42

3.2.17 Amplex™ red cholesterol assay 42

3.2.18 Proteome profiler human phospho-kinase array 42

3.2.19 Measurement of hormone levels 43

3.2.20 Statistical analysis 43

CHAPTER 4

The Discovery of a Regulatory Role of Prohibitin-1 in Testosterone Production

Prohibitin-1 plays a regulatory role in Leydig cell steroidogenesis...44

Graphical abstract...45

Highlights.. 45

4.0 Summary 46

4.1 Introduction.. 47

4.2 Materials and Methods.. 49

4.3 Results... 50

4.3.1 Male mPHB-1 mice display smaller testes, elevated serum testosterone and lower gonadotropin levels 50

4.3.2 PHB1 and mPHB1 mice overexpress PHB1 in their Leydig cells 54

4.3.3 PHB1 is a LH-regulated protein and plays a role in hormone production by Leydig cells 56

4.3.4 Leydig cells from PHB1 and mPHB1 mice showed distinct mitochondrial and lipid droplet characteristics...60

4.3.5 PHB1 contains putative cholesterol binding motifs and interacts with cholesterol…65

4.3.6 PHB1 interacts with StAR and P450scc and forms a heterodimeric megacomplex with PHB2 in Leydig cells..67

4.3.7 PHB1 modulates PKA and ERK signaling in Leydig cells.......................................70

4.4 Discussion 77

Supplementary Figure 1...82

BRIDGE TO CHAPTER 5..83

CHAPTER 5

Exploring the Potential Role of the Intracellular Cholesterol Pool in Steroidogenic Cells

The intracellular cholesterol pool in steroidogenic cells plays a role in basal steroidogenesis.. 84

Highlights….. 84

5.0 Abstract 85

5.1 Introduction 86

5.2 Materials and Methods.. 88

5.3 Results... 88

5.3.1 Culturing steroidogenic cells in a lipoprotein-depleted medium leads to the depletion of the intracellular cholesterol pool & lipid droplets 88

5.3.2 Ultrastructural analysis of steroidogenic cells after the manipulation of the intracellular cholesterol pool..92

5.3.3 Cholesterol deprivation induces steroidogenic events/factor in Leydig cells...........96

5.3.4 The role of cholesterol in basal steroidogenesis is conserved in adrenocortical and placental cells 101

5.3.5 CD-induced changes sustain basal steroidogenesis..114

5.4 Discussion 116

CHAPTER 6

Conclusion, limitations, and future directions...120

6.1 Conclusion..120

6.2 Limitations 122

6.3 Future Directions..123

CHAPTER 7

References 125

List of Publications 146

LIST OF FIGURES

Chapter 1

Figure1.1 Similarities and differences between major steroidogenic cells in relation to physiological demand and cellular structure pertaining to lipid droplets and mitochondrial structure/content. 4

Figure1.2 Schematic diagram depicting known and potential relationship between cholesterol homeostasis and mitochondrial attributes in major steroidogenic cells. 5

Figure 1.3 Schematic diagram depicting known (solid arrow) and potential (dashed arrow) interplay between hormone- and metabolic status-induced autophagy/lipophagy in a steroidogenic cell 22

Figure 1.4 Schematic diagram depicting mitochondria, autophagy and lipid metabolism/scaffold-related known attributes of PHB 25

Chapter 4

Graphical abstract 45

Figure 4.1 Male mPHB1 mice display reduced fertility, smaller testis, elevated testosterone and lower gonadotropin levels 51

Figure 4.2 PHB1 is a LH-regulated protein and plays a role in hormone production by Leydig cells 58

Figure 4.3 Leydig cells from PHB1- and mPHB1 mice showed distinct mitochondrial and lipid droplet characteristics..62

Figure 4.4 PHB1 contains putative cholesterol binding motifs, interacts with StAR and P450scc, and forms heterodimeric megacomplex with PHB2 in Leydig cells..66

Figure 4.5 PHB1 modulates PKA and ERK signaling in Leydig cells..71

Figure 4.6 Phosphokinase array profiling of PHB1 and mPHB1 transfected MA-10 cells in response to hCG.. 73

Figure 4.7 Steroidogenic markers are upregulated in the testis from PHB1 transgenic mice and PHB1 modulates ERK phosphorylation in a context-dependent manner......................................75

Supplementary figure 1. Immunoblots showing the shRNA- and CRISPR/Cas9-mediated knockdown of PHB1 in MA-10 cells…..82

Chapter 5

Figure 5.1 Culturing steroidogenic cells in lipoprotein-depleted medium leads to depletion of intracellular cholesterol pool...90
Figure 5.2 TEM analysis of MA-10 cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition..93
Figure 5.3 TEM analysis of Y-1 cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition..94
Figure 5.4 TEM analysis of BeWo cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition 95
Figure 5.5 Intracellular cholesterol depletion induces steroidogenic factors and events in MA- 10 cells 99
Figure 5.6 Intracellular cholesterol depletion induces steroidogenic factors and events in Y-1 cells 103
Figure 5.7 Intracellular cholesterol depletion induces steroidogenic factors and events in BeWo cells 106
Figure 5.8 Intracellular cholesterol depletion induced in non-steroidogenic H9c2 cells…..........107
Figure 5.9 Ultrastructural analysis of MA-10 and Y-1 cells after augmenting intracellular cholesterol pool 109
Figure 5.10 Changes in protein levels of autophagy, mitochondrial dynamics and steroidogenic markers in response to StAR knockdown-mediated increased accumulation of lipid droplets/intracellular cholesterol pool in MA-10 and Y-1 cells..112
Figure 5.11 Cholesterol deprivation-induced changes in steroidogenic cells sustain basal steroidogenesis 115
Figure 5.12 Cholesterol – more than a substrate for steroid hormones.......................................118

LIST OF TABLES

Chapter 3

3.1 Key resource table..33

LIST OF OPEN ACCESS AND COPYRIGHT MATERIALS

Two out of three articles used in the thesis are published in open access journals (i.e., in *Cells* and *iScience),* which are reproduced here under the CC BY license ((http://creativecommons.org/licenses/by/4.0/). The publisher of the third article (*The Journal of Steroid Biochemistry and Molecular Biology* by Elsevier) allows the author to retain right to include the published work in a thesis or dissertation and a permission is not required.

LIST OF ABBREVIATIONS

%	Percent
°C	Degree celcius
µl	Microlitre
3β-HSD	3-beta-hydroxysteroid dehydrogenase
Ab	Antibody
ACTH	Adrenocorticotropic hormone
Atg7	Autophagy related 7
BSA	Bovine serum albumin
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
CD	Cholesterol-depleted
CO_2	Carbon dioxide
Cyp11B1 or B2	11β- hydroxylase and aldosterone synthase
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's minimal essential media
Drp1	Dynamin-associated protein 1
E2	Estradiol
eCG	Equine chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FABP4	Fatty acid binding protein 4
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
g	Gram
GCs	Granulosa cells
h	Hour
hCG	Human chorionic gonadotropin
HCl	Hydrochloric acid

HDL	High-density lipoprotein
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IMM	Inner mitochondrial membrane
LC	Leydig cells
LC3	Microtubule-associated protein 1A/1B-light chain 3
LD	Lipid droplet
LDL	Low-density lipoprotein
LH	Luitinizing hormone
M	Molar
MAPK	Mitogen-activated protein kinase
MEC2	Mechanosensory protein2
Mfn1	Mitofusin1
Mfn2	Mitofusin2
Min	Minute(s)
MLN64	Metastatic lymph node 64
NC	Normal culture
OD	Optical density
OMM	Outer mitochondrial membrane
Opa1	Optic atrophy 1
P4	Progesterone
P450scc/Cyp11A1	Cytochrome P450 side chain cleavage enzyme
PBS	Phosphate buffer saline
PHB/PHB1	Prohibitin/prohibitin1
PHB2/REA	Prohibitin-2/repressor of estrogen activity
PKA	Protein kinase A
PM	Plasma membrane
PTMs	Post-translational modifications
RNA	Ribonucleic acid

SC	Steroidogenic cell
Sec	Seconds
SER	Smooth endoplasmic reticulum
Ser	Serine
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SR-B1	Scavenger receptor class B type1
StAR	Steroidogenic acute regulatory protein
START	StAR-related transfer domain
STARD3	StAR-related lipid transfer domain containing 3
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TG	Triglycerides
WT	Wild type

CHAPTER 1

INTRODUCTION

Sections in this chapter are published as a review article titled:

"The Expanding Role of Mitochondria, Autophagy and Lipophagy in Steroidogenesis"

Geetika Bassi, Simarjit Kaur Sidhu, and Suresh Mishra

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1.0 Abstract

The fundamental framework of steroidogenesis is similar across steroidogenic cells, especially in initial mitochondrial steps. For instance, the START domain containing protein-mediated cholesterol transport to the mitochondria, and its conversion to pregnenolone by the enzyme P450scc, is conserved across steroidogenic cells. The enzyme P450scc localizes to the inner mitochondrial membrane, which makes the mitochondria essential for steroidogenesis. Despite this commonality, mitochondrial structure, number, and dynamics vary substantially between different steroidogenic cell types, indicating implications beyond pregnenolone biosynthesis. This review aims to focus on the growing roles of mitochondria, autophagy and lipophagy in cholesterol uptake, trafficking, and homeostasis in steroidogenic cells and consequently in steroidogenesis. We will focus on these aspects in the context of the physiological need for different steroid hormones and cell-intrinsic inherent features in different steroidogenic cell types beyond mitochondria as a mere site for the beginning of steroidogenesis. The overall goal is to provide an authentic and comprehensive review on the expanding role of steroidogenic cell-intrinsic processes in cholesterol homeostasis and steroidogenesis, and to bring attention to the scientific community working in this field on these promising advancements. Moreover, we will discuss a novel mitochondrial player, prohibitin-1 (PHB1), and its potential role in steroidogenic mitochondria and cells, and consequently, in steroidogenesis.

1.1 Introduction

Steroid hormones are an important class of regulatory molecules, which are synthesized mainly in the adrenal glands, the ovary, and the testis, in response to steroidogenic stimuli, and regulate growth and drive a variety of physiological processes, such as reproduction and metabolism [Saha et al., 2021]. The importance of steroid hormones is evident from their wide-ranging essential functions in the body physiology, including carbohydrate metabolism, stress response, and in the regulation of salt balance pertaining to the maintenance of blood pressure by adrenal corticoids to the role of sex steroid hormones in males and females in the development of secondary sex characteristics, maintenance of reproductive functions, and perpetuation of life, as well as an essential role of progesterone for a successful pregnancy [Miller and Bose, 2011]. The steroid hormones can be distinguished from one another by their diverse physiological actions in the body; however, an overarching commonality among them is that they all are produced from cholesterol. Thus, an advanced understanding of steroid hormone biology is a requisite in the biomedical field.

The first step in the biosynthesis of steroid hormones is the enzymatic cleavage of a six-carbon unit side chain of cholesterol molecule by the 20–22 desmolase/lyase activity of the cytochrome P450 side chain cleavage (P450scc) enzyme system located in the inner mitochondrial membrane (IMM) [Rone et al., 2009 and Lin et al., 2016]. Steroidogenesis is a finely compartmentalized, multistep enzymatic process in steroidogenic cells, which involve different cellular compartments, including the cytoplasm, mitochondria, and the smooth endoplasmic reticulum (SER). The initiation of steroidogenesis that involves the enzymatic cleavage of the cholesterol side chain is conserved across steroidogenic cells. The enzyme P450scc localizes to the matrix side of the IMM [Rone et al., 2009 and Lin et al., 2016], which makes the mitochondria central to steroidogenesis. Thus, it is not surprising that steroidogenic cells (e.g., adrenocortical cells in the adrenal glands, the granulosa, and theca cells in the ovary, Leydig cells in the testis, and syncytial trophoblast cells in placenta) are rich in mitochondria [Medar et al., 2020; Castillo et al., 2015 and Papadopoulos et al., 2012]. In this review article, we will refer to them as 'steroidogenic mitochondria' because of their inherent ability to initiate steroidogenesis not shared by the mitochondria from non-steroidogenic cells. For instance, steroidogenic acute regulatory protein (StAR)-mediated cholesterol transport to the mitochondria and its subsequent utilization by the enzyme P450scc. As mitochondria is an important signaling hub, it is likely that this special

attribute of steroidogenic mitochondria might drive many aspects of steroidogenesis in a steroidogenic cell type-specific manner because the physiological demand for each steroid hormone varies substantially. Such a difference in steroid hormone levels in the body's physiology may explain why the structure, number and distribution of mitochondria vary substantially across steroidogenic cells (Figure 1.1).

Adrenocortical cells

- Involved StAR and acutely regulated
- Regulated by pituitary-derived trophic hormone
- Prominent lipid droplets
- Higher mitochondrial density
- High and continuous physiological demand

Leydig cells

- Involved StAR and acutely regulated
- Regulated by pituitary-derived trophic hormone
- Prominent lipid droplets
- Higher mitochondrial density
- Continuous physiological demand

Granulosa and theca cells

- Involved StAR and acutely regulated
- Regulated by pituitary-derived trophic hormones
- Prominent lipid droplets
- Relatively low mitochondrial density
- Cyclic physiological demand

Syncytial trophoblast cells

- Lack StAR and acute regulation
- Not regulated by pituitary-derived trophic hormones
- Utilize substrate derived from other steroidogenic tissues
- Poorer in LDs and mitochondria than other steroidogenic cells
- During pregnancy only

Figure 1.1. Similarities and differences between major steroidogenic cells in relation to physiological demand and cellular structure pertaining to lipid droplets and mitochondrial structure/content.

For example, mitochondrial features and the distribution in adrenocortical cells and Leydig cells are relatively more prominent in comparison to ovarian and placental steroidogenic cells [Kraemer et al., 2017]. While such differences in mitochondria in steroidogenic cells may be explained on the basis of their steroidogenic capacity and the physiological need of different steroid hormones, which ranges from picomoles to micromoles (Figure 1.1), it is also possible that a substantial difference in mitochondrial attributes in different steroidogenic cells are a reflection

of their need to maintain the cholesterol homeostasis required to maintain basal, acute, and chronic steroidogenesis in a steroidogenic cell type-specific manner.

The steroid hormones are not stored in secretory vesicles like peptide hormones but released into the blood upon their biosynthesis [Kraemer et al., 2017]. This instant set-up between the biosynthesis and release of steroid hormones is expected to require an arrangement to maintain the readily available cholesterol pool within steroidogenic cells, because the cholesterol contents of mitochondrial membranes, especially the IMM, where steroidogenesis begins, is insufficient to support steroidogenesis [Kraemer et al., 2017]. Emerging evidence suggest that mitochondrial dynamics, autophagy, and related lipophagy play crucial roles in intracellular cholesterol uptake and in the maintenance of cholesterol homeostasis in steroidogenic cells, and consequently support steroid hormone production to maintain physiological functions (Figure1.2).

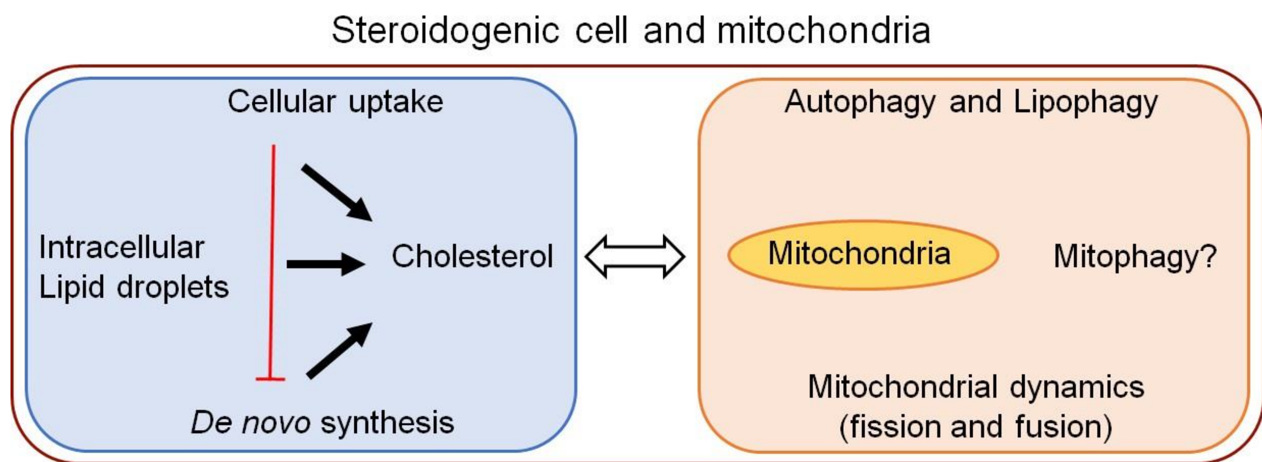


Figure1.2. Schematic diagram depicting known and potential relationship between cholesterol homeostasis and mitochondrial attributes in major steroidogenic cells. The interplay between different intrinsic factors is expected to vary under basal, acute, and chronic steroidogenic states (as applicable) because of a wide range of different steroid hormone levels and their physiological needs.

Thus, an expanding role of cell-intrinsic processes (e.g., autophagy, lipophagy and mitochondrial attributes) in steroidogenesis have created a need for a timely review article for the benefit of the scientific community engaged in this field, and to facilitate research to answer fundamental and emerging unanswered questions. In this review, we will focus on the cell-intrinsic attributes of different steroidogenic cells involved in cholesterol handling and mitochondrial activities. We have two interconnected goals: first, to discuss the importance of various processes involved to maintain a readily available pool of cholesterol for the varying need of steroidogenic

demands, and second, to review the growing role of mitochondria, autophagy, and lipophagy, and other related activities in a steroidogenic cell type-specific manner to meet the diverse physiological demand of each steroid hormone (Figure1.2). While several excellent review articles are available on receptor mediated cholesterol uptake (e.g., SR-B1 and LDL receptors), cholesterol mobilization and transport to mitochondria in steroidogenic cells [Miller and Bose, 2011; Rone et al., 2009; Lin et al., 2016; Medar et al., 2020; Castillo et al., 2015; Papadopoulos et al., 2012 and Kramer et al., 2017], to the best our knowledge there is a lack of review articles in current literature, which provide a broader perspective in this context integrating the accruing role of autophagy, lipophagy and mitochondrial attributes in relation to steroidogenesis, which is the focus of this review. In addition, we will discuss a novel mitochondrial player, prohibitin1 (PHB1, also known as PHB), and its potential role in integrating steroidogenic mitochondria with cholesterol handling and steroidogenesis in a context-dependent manner based on our current knowledge of PHB1 and the PHB family of proteins in mitochondrial biology and lipid metabolism. In this review, we will not discuss signaling and cellular events in cholesterol sequestering and trafficking in steroidogenic cells but instead will focus on accruing evidence related to autophagy, lipophagy, and the mitochondrial dynamics involved in handling the cholesterol pool in the cytoplasm of steroidogenic cells and highlight any pertinent questions that may arise in this exploration.

1.2 Steroidogenic Cells and Steroidogenic Mitochondria

A cell is classified as “steroidogenic” if it expresses the enzyme P450scc and therefore can catalyze the first reaction of steroidogenesis (i.e., the conversion of cholesterol to pregnenolone). Many cells can transform steroids produced in other cells (e.g., adipocytes), but only cells expressing P450scc are steroidogenic [Miller and Bose, 2011,]. As P450scc resides in the IMM, the mitochondria of steroidogenic cells can be called steroidogenic mitochondria by the same token, because of their distinct ability to begin steroidogenesis (as mentioned earlier), which is not shared by the mitochondria from non-steroidogenic cells. However, as IMM is cholesterol-poor, there is a prerequisite for steroidogenesis by P450scc, i.e., for a cholesterol import to the mitochondria, which is mediated through the START domain containing protein (e.g StAR in steroidogenic adrenocortical and gonadal cells and metastatic lymph node 64 (MLN64, also known as STARD3) protein in placental cells [Miller and Bose, 2011,]. Thus, P450scc is essential, but

not sufficient, to initiate steroidogenesis by itself because of its physical location in the IMM and is assisted by proteins involved in cholesterol transport to the mitochondria. Consequently, unlike many metabolic pathways (e.g., glycolysis, citric acid cycle, fatty acid synthesis), the committed step in steroidogenesis is not an enzymatic reaction (i.e., the P450scc- mediated conversion of cholesterol to pregnenolone, which was previously thought to be one), but rather, cholesterol transport to the mitochondria that is mediated by the StAR protein [Miller and Bose, 2011,]. In addition to P450scc, 11-hydroxysteroid dehydrogenase and aldosterone synthase in adrenocortical cells localize to the mitochondrial IMM, and 3β-hydroxysteroid dehydrogenase has been reported to be present in the mitochondria [Chapman et al., 2005]. In fact, 3β-HSD was first isolated from the mitochondria [Simard et al., 2005]. Furthermore, the mitochondria in steroidogenic cells are responsive to the actions of trophic hormones, and steroidogenic cells have evolved a variety of ways to acquire and mobilize cholesterol for the maintenance of basal, acute, and chronic steroidogenesis. Thus, it is a combination of interconnected features spanning different cellular compartments, which define the cell type-specific function of a steroidogenic cell and the steroidogenic mitochondria (Figure1.2).

The precursor cholesterol for steroidogenesis is known to come from at least three sources, such as the mobilization of cholesterol from the lipid droplets (LDs), the uptake of circulating cholesterol esters, and the de novo synthesis of cholesterol, which have been described extensively in many review articles [Rone et al., 2009 and Azhar, 2003]. However, our understanding of the relative contributions of various sources of cholesterol to the different stages of steroidogenesis (i.e., basal, acute, and chronic) in major steroidogenic cells remains limited. It is likely that these processes work in a coordinated manner to maintain the physiological needs of different steroid hormones (which vary substantially) in a context-dependent manner. For example, the mobilization of cholesterol from LDs may play a major role in the acute response to trophic hormones, whereas the de novo synthesis of cholesterol may be a major contributor for basal and chronic steroidogenesis, and in the cellular uptake of cholesterol in replenishing the depleted, readily available pool due to the acute response, and in the maintenance of a chronic response. Similarly, the instantaneous contribution of cellular uptake and the de novo synthesis of cholesterol for acute steroid production in response to trophic hormones is expected to be minimal (Figure1.2).

In addition to steroidogenic cells, the scavenger receptor class B type I (SR-BI) that are involved in cellular cholesterol uptake are found in many non-steroidogenic cell types, such as macrophages and endothelial cells [Connelly et al., 2003]. In testicular interstitium, the predominant cell types are Leydig cells and macrophages, and both cell types appear to be dependent on each other. A decrease in the number of one cell type (by genetic or pharmacological approaches) leads to a corresponding decrease in other cell types and vice versa [Rone et al., 2009 and Azhar, 2003]. In addition, testicular macrophages have been implicated in supporting Leydig cell steroidogenesis, especially in bypassing the StAR-mediated cholesterol transport by providing 25-hydroxysteroid cholesterol to the Leydig cells [Heinrich et al., 2020]. In addition, the potential role of phagocytic activities of macrophages in cholesterol recycling in testicular interstitium (and potentially in other steroidogenic tissues, such as adrenals and ovaries) may not be discounted.

1.2.1 Steroidogenesis and Mitochondrial Structures—The Role of Steroidogenic Enzymes

Structural changes in the mitochondria appear to be an integral feature of the differentiation of steroidogenic cells, which involve an acquisition of steroidogenic capability due to the expression of steroidogenic enzymes during development [Farkash et al., 1986 and Chien et al., 2013]. For instance, the differentiation of non-steroidogenic cytotrophoblasts into steroidogenic syncytial trophoblasts during the development of placenta involves increased expression of P450scc, which coincide with structural changes in the mitochondria. This includes a reduction in mitochondrial size and a change in the shape of mitochondrial cristae [Martinez et al., 1997]. A difference in mitochondrial cristae shape has also been reported between non-steroidogenic cells of adrenal medulla and steroidogenic adrenocortical cells [Crivellato et al., 2004]. Interestingly, the mitochondrial cristae structure and intercristal space also differ between different steroidogenic cells of the adrenal cortex [Farkash et al., 1986 and Chien et al., 2013]. For example, mitochondrial cristae are lamellar with a wide intercristal space in aldosterone producing zona glomerulosa, whereas they are vesicular in cortisol/corticosterone producing the zona fasciculata [Chien et al., 2013]. Moreover, the different steroidogenic cells of the ovary also display differences in their mitochondrial cristae structures. In granulosa cells, the mitochondria have an elongated shape with lamellar cristae, whereas in luteal cells, the mitochondria are spherical in shape with tubular-vesicular cristae [Chien et al., 2013]. As the steroidogenic capacity (and corresponding mitochondrial attributes) of different steroidogenic cells vary substantially because of their diverse

physiological need, their relationship with mitochondrial shape and cristae structure would imply that steroidogenic enzymes in the mitochondria may have a role in controlling mitochondrial structure and function. The findings from transgenic mice that overexpress Cyp11a1, as well as *StAR* and *Cyp11A1* knockout mouse models are consistent with this notion. For instance, the mitochondria of the luteal cells of the Cyp11A1 transgenic mouse model are elongated from their normal spherical shape [Chien et al., 2013]. Furthermore, the impact of *Cyp11A1* and *StAR* knockout on the mitochondria of zona fasciculata cells differ from each other, which is more severe in the former than the later [Haung et al., 2012 and Ishii et al., 2002]. As Cyp11a1 and StAR work tandemly in the beginning of steroidogenesis and their knockout models display overlapping phenotypes, their differential effect on mitochondrial phenotype in steroidogenic cells are likely due to the direct effect of gene deficiency rather than secondary to dysregulation of cholesterol handling. In this context, it is important to note that placental and ovarian steroidogenic cells undergo proliferation and differentiation in each cycle, which is not the case with the adrenals and testis; thus, the workload and related mitochondrial attributes are expected to vary substantially to meet their respective physiological demands.

1.2.2 Hormone-Induced and Cell-Intrinsic Processes in Steroidogenic Cells

Tropic hormone-induced steroidogenesis in the adrenals and the gonads have been studied extensively. However, the context-dependent potential contributions of cell-intrinsic events remain largely unexplored. New evidence demonstrating the importance of autophagy and lipophagy in steroidogenesis in combination with our previous knowledge of cholesterol homeostasis and mitochondrial dynamics in steroidogenic cells have created an opportunity to explore these aspects and attain new insights. For example, preclinical models mimicking congenital adrenal hyperplasia (CAH) phenotype provides an excellent example to investigate cell-intrinsic processes and the physiological needs of cell type-specific steroidogenesis on the varying impacts of the loss-of-function mutation in StAR [Caron et al., 1997 and Mullins et al., 2009]

The characteristic features of lipoid CAH in humans are hypertrophied adrenals with enlarged lipid droplets because of high ACTH level and renin activity, and substantially reduced serum levels of steroid hormones [Miller, 1997]. Bose et al. [1996] proposed a two-hit model by identifying mutations in StAR to explain the pathophysiology of lipoid CAH in humans. According to this model, the loss of StAR activity due to naturally occurring mutation is the first hit, causing reduced steroidogenesis and consequently an increase in trophic hormones (i.e.,

ACTH, LH and FSH) [Miller, 1997]. Higher trophic hormones lead to increased production of secondary messenger and consequently increased cholesterol uptake and biosynthesis by adrenocortical cells. This imbalance in cholesterol accumulation and utilization leads to the second hit—mitochondrial damage due to lipotoxicity and the loss of residual steroidogenic capacity [Bose et al., 1996], which explains the phenotypic manifestation in different steroidogenic cell types/tissues. Thus, a balance between the cholesterol availability and steroidogenesis is critical for the normal functioning of steroidogenic cells. For instance, earlier manifestation of the loss of StAR function in steroidogenic cells of the fetal testes and after birth in adrenocortical cells correlates with the initiation of steroidogenesis in both tissues, respectively [Miller, 1997]. On the other hand, the fetal ovary does not make steroids and remains unstimulated and therefore remains normal until puberty. In aggregate, this evidence suggests that coordination between the upstream stimuli and downstream functions are important to maintaining mitochondrial functions in steroidogenic cells. Of note, the *StAR*-knockout mouse model recapitulates the phenotype of StAR deficiency in humans. Thus, the phenotypic manifestation of StAR deficiency precisely correlates with the sensitivity of steroidogenic cells to respective tropic hormones and their steroidogenic activity during different stages in life. In summary, there is much to learn from the comparative accounts of steroidogenesis in different steroidogenic cells.

1.2.3 The Importance of Mitochondrial Dynamics in Steroidogenesis

The initiation of the biosynthesis of steroid hormones occurs in the mitochondria, which are known to undergo dynamic changes called mitochondrial fission and fusion [Park et al., 2019]. Therefore, it is likely that the cellular and molecular changes in mitochondria would influence steroidogenesis. Indeed, many studies have shown that mitochondrial dynamic changes are closely associated with the biosynthesis of steroid hormones in steroidogenic cells [Medar et al., 2020; Castillo et al., 2015 and Papadopoulos et al., 2012]. For example, cAMP-induced steroid hormone production has been reported to be accompanied by increased mitochondrial mass [Park et al., 2019], specifically an increase in mitochondrial fusion, whereas a reduction occurs in mitochondrial fission. Among the mitochondrial proteins that are involved in shaping the mitochondria, dynamin-associated protein 1 (Drp1) level was found altered in response to dibutyryl-cAMP (db-cAMP) stimulation. Particularly, an increase in the phosphorylation of Drp1 at Ser^{637} correlated with steroid hormone production in the primary adult rat Leydig cells and in a

model murine cell line of Leydig cells. In addition, gonadotropin administration was found to alter the status of Drp1 phosphorylation in the Leydig cells isolated from immature rat testes [Park et al., 2019]. Overall, mitochondrial dynamics at large were found to be directly linked to steroidogenesis, and Drp1 was found to play an important regulatory role during steroidogenesis [Park et al., 2019]. Thus, cAMP-PKA pathway, which plays a central role in the Leydig cell steroidogenesis is also involved in the regulation of mitochondrial dynamics to facilitate steroidogenesis. Moreover, hypogonadism was found to affect mitochondrial fusion proteins such as mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) in the Leydig cells by reducing the transcription of mitochondrial fission protein Drp1, as well as Mfn1 and Mfn2, without changing mitochondrial dynamin like GTPase protein optic atrophy 1 (Opa1) levels [Park et al., 2019].

The differences between placental trophoblasts and syncytiotrophoblasts that sustain progesterone production during human pregnancy is accompanied by mitochondrial fragmentation and cristae remodeling [Vangrieken et al., 2021]. Subsequent work revealed that the mitochondria shaping Opa1 controls the efficiency of steroidogenesis in placental cell line: BeWo cells [Vangrieken et al., 2021]. This finding further supports the notion that structural changes in mitochondria play a role in steroidogenesis. However, a similar change in mitochondrial structure has not been reported in other steroidogenic cell types, which express StAR for cholesterol transport to the mitochondria.

1.2.4 Steroidogenic Mitochondria—A Comparative Account

While the final steroid hormone product differs in different steroidogenic cell types, the first step in the steroidogenic pathway is precisely similar, which is catalyzed by the enzyme P450scc located in the IMM. In addition to the common first step of the steroidogenic pathway, the final steps in the biosynthesis of glucocorticoids and mineralocorticoids are also catalyzed by two closely related mitochondrial enzymes: CYP11B1 and CYP11B2 (11β-hydroxylase and aldosterone synthase), located in the IMM [Mornet et al., 1989]. Both enzymes display differential expression in three different steroidogenic cell types in the adrenal cortex, which in turn contribute to cell type-specific corticosteroid production. Moreover, 3β-hydroxysteroid dehydrogenase has also been reported to localize to the mitochondria [Miller, 2013]. In this context, it is important to note that three different steroidogenic cell types of the adrenal cortex, comprising of three different zones (i.e., the glomerulosa, fasciculata and reticularis) display morphological differences in photomicrographs, which are easily distinguishable from each other. However, it remains unclear

what contributes to such morphological differences, despite their common functions (i.e., the production of the steroid hormone). It is likely that the physiological demand of the steroid hormone they produce, and the corresponding requirements of the steroidogenic machinery (e.g., the expression levels of steroidogenic enzymes involved, mitochondrial numbers, and the readily available cholesterol pool) contribute to such differences. It has been suggested that mitochondrial steroidogenic enzymes play a role in the regulation of mitochondrial morphology and structure, which in turn influence steroid production [Chein et al., 2017]. For example, mitochondrial cristae in steroidogenic cells are vesicular and/or tubular in shape, which relate to the degree of their steroidogenic function [Chein et al., 2017].

Steroidogenic mitochondria, particularly in adrenal and gonadal steroidogenic cells, are affected differently by the actions of the pituitary tropic hormones during the acute and the chronic response [Orme-Johnson, 1990]. The acute response begins in minutes after the binding of the pituitary tropic hormone to their cognitive receptors. This is accomplished by increased trafficking of cholesterol from the cytoplasmic compartment to P450scc in the IMM and does not involve change in the levels of proteins involved in catalyzing this enzymatic step [Orme-Johnson, 1990], whereas the longer time effects involve upregulation of the protein levels of steroidogenic enzymes. Thus, the control of steroidogenesis by the mitochondrion itself is exerted at two levels: first, the regulation of cholesterol pool as a precursor substrate, and second, the regulation of the mitochondrial import and processing of the nuclear transcribed steroidogenic enzymes, including 11β-dehydrogenase and aldosterone synthase for adrenal steroidogenesis, which may vary under basal and stimulated states. An important point that needs to be considered in this context is the potential consequences of the NADPH-utilizing metabolic reactions on portioning of NADH for the respiratory chain and on the proton gradient, which is expected to vary between the adrenocortical and other steroidogenic cells. This is because of differences in the number of steroidogenic enzymes present in the IMM in adrenocortical cells and other steroidogenic cells, and differences in the physiological levels of the respective steroid hormones produced. These differences are expected to create mitochondrial heterogeneity between steroidogenic cells, which is a topic that currently remains unclear to us. Furthermore, the mitochondria in steroidogenic cells not only have enzymes for steroidogenesis, but also have unique mechanisms for regulating cholesterol availability of these enzymes, regulation of StAR levels and cholesterol trafficking.

Structural changes in steroidogenic mitochondria have been reported during the development and differentiation of steroidogenic cells. For instance, the differentiation of cytotrophoblasts and syncytiotrophoblasts is marked by a substantial increase in the expression levels of CYP11A1 and vesicular cristae [Martinez and Strauss, 1997]. As discussed in the Section 1.2.2, a correlation between mitochondrial structure and steroidogenesis has also been reported in adrenocortical cells and ovarian granulosa and theca cells [Farkash et al., 1986 and Chein et al., 2013]. It is possible that steroidogenic enzymes in the mitochondria play a role in regulating mitochondrial attributes in steroidogenic cells [Chein et al., 2017]. Thus, the relationship of mitochondrial structure and steroidogenesis appears to be two-ways in steroidogenic cells, as hormone-induced steroidogenesis leads to changes in mitochondrial structure and dynamics, whereas manipulation of the mitochondrial remodeling affect steroidogenesis [Chein et al., 2017 and Wasilewski et al., 2012], which in turn feedback to steroidogenic stimuli and creates a regulatory cycle. For example, when the level of OPA1 in BeWo cells (a model human placental cell line) is reduced, cholesterol flux into mitochondria and steroid production are increased [Wasilewski et al., 2012]. Thus, there are two specific aspects of the mitochondria in steroidogenic tissues, including mechanisms to deliver cholesterol to the mitochondria for steroidogenesis, and mitochondrial enzymes in the inner mitochondrial membrane for the initiation of steroidogenesis [Miller, 2013].

In addition to the P450scc that is present in all steroidogenic cells, adrenocortical mitochondria contain two additional P450 enzymes: 11β–hydroxylase in zona fasciculata and aldosterone synthase in zona glomerulosa, which also localize to the IMM. The former catalyzes the conversion of 11-deoxycortisol to cortisol, while the latter catalyzes the conversion of deoxycorticosterone to aldosterone [White et al., 1994; Fardella and Miller, 1996 and Miller and Auchus, 2011]. All three steroidogenic enzymes that are localized to the IMM use reducing equivalents provided via ferredoxin reductase and ferredoxin. Thus, a demand for reducing equivalents in adrenocortical cells is expected to be much higher than other steroidogenic cell types in the gonads and their high demand in adrenocortical cells may compete with non-steroidogenic processes that use them. It is likely that these differences in the needs of different steroidogenic cells would affect mitochondrial attributes differently and may contribute to differences in mitochondrial structure, number and function in different steroidogenic cell types and tissues.

1.3 Placental Steroidogenesis—What We Can Learn from the Similarities and Differences with Adrenal and Gonadal Steroidogenesis?

Despite several commonalities with adrenal and gonadal steroidogenesis (e.g., ability to synthesize steroid hormones by specific cell types), placental steroidogenesis displays certain unique arrangements and characteristics, which are shared among placentae from different species. These include differences in regulatory mechanisms that control the expression of steroidogenic enzyme genes from other steroidogenic tissues, an interplay between maternal and fetal compartments to support placental steroidogenesis, and the regulatory influences on maternal hypothalamic-pituitary-gonadal axis and fetal adrenal corticosteroids [Strauss et al., 1996]. Importantly, the ability to metabolize steroid hormones derived from the maternal ovary and fetal adrenal is one common feature of trophoblast cells, despite the marked differences in placental morphologies in different species. Thus, the cell-intrinsic steroidogenic characteristics of trophoblasts, particularly cholesterol handling and mitochondrial attributes, are expected to vary substantially from those of the ovarian granulosa and theca cells, as well as testicular Leydig cells. A fitting example of this is the lack of StAR expression and acute steroidogenesis in the placenta, which are characteristic features of adrenocortical and gonadal steroidogenesis. Due to this fundamental difference between the two steroidogenesis types, it is likely that the mechanisms involved in cholesterol handling and mitochondrial activities between them will also vary substantially. For example, the regulatory mechanisms involved in controlling the expression of the placental P450 gene are different than in the adrenal cortex and gonads. In addition, it appears that the second messenger cAMP, which plays a central role in adrenal and gonadal steroidogenesis, does not have a prominent role in regulating placental steroidogenesis in many species [Strauss et al., 1996]. Moreover, the trophoblast 3β-HSD is different from that which is expressed in the gonads and adrenal cortex, and the placental aromatase gene is transcribed from unique promoters [Strauss et al., 1996]. Furthermore, unlike adrenals and gonads, which synthesize steroid hormones from the precursor cholesterol molecule, the placenta is able to utilize steroid precursors contributed by both the mother and the fetus, and are influenced by their hormones. Substantial evidence exists in literature to suggest that the placenta engages in a dynamic steroid-mediated dialogue with both the maternal pituitary and ovary and the fetal adrenal cortex [Strauss et al., 1996]. The unique aspects of placental steroidogenesis mentioned above raise many important biological questions with unclear answers (see Appendix A. Outstanding Questions).

Like the adrenal cortex and the gonads, progesterone production by the human placenta requires pregnenolone synthesis from cholesterol by cytochrome P450scc [Tuckey et al., 2004; Morel et al., 2016; Tuckey et al., 1994 and Strauss et al., 2000]. Most steroidogenic tissues use StAR protein to deliver cholesterol to the IMM where P450scc resides. However, instead of StAR, the human placenta expresses STARD3 (MLN64), which has a C-terminal domain homologous to StAR [Tuckey et al., 2004, and Morel et al., 2016]. Many studies have shown that the cholesterol binding domain of both proteins in humans have similar biophysical and functional properties and are able to support steroidogenesis in placental tissue and their cell derivatives in different species [Conley et al., 1992; Tuckey et al., 2002; Conley et al., 1992; Draycott et al., 2020; Watari et al., 1997; Bose et al., 2000; Tsujishita et al., 2000; Soccio et al., 2002; Romanowski et al., 2002; Zhang et al., 2002; Arakane et al., 1996 and Bose et al., 2002].

A unique aspect of placental steroidogenesis is its close relationship with maternal and fetal steroidogenesis. For example, E2 (estradiol) and P4 (progesterone) produced from maternal ovaries play important roles in placenta formation and function during early stages of pregnancy. Subsequently during later stages, the placenta itself acquires the ability to produce the P4 required to maintain pregnancy [Hong et al., 2019]. In this context, it is important to note that while the E2 and P4 produced from the maternal ovaries are under the control of maternal pituitary gonadotropins, this is not the case with placental P4 production. In addition, the placenta also utilizes steroidogenic metabolites of the fetal adrenal glands [Hong et al., 2019]. It is likely that because of this arrangement, placental steroidogenesis would not require intracellular arrangements and regulatory mechanisms (or would substantially vary from) that are required for steroidogenesis in other steroidogenic tissues (i.e., adrenal, ovary and testis). Pregnancy in women is marked by substantial changes in their endocrine system [Costantine, 2014]. For instance, levels of E2 and P4 dramatically increase during pregnancy, suppressing the hypothalamic-pituitary axis and subsequently the ovarian and menstrual cycle [Kurnar and Magon, 2012]. Such a shift in the production of steroid hormones would require an enhanced expression and activity of the steroidogenic enzymes in the placental tissue, resulting in increased serum and placenta levels of E2 and DHEA near the end of gestation.

Many placental species, including humans, do not express 17α-hydroxylase [Conley et al., 1992; Albrecht and Pepe, 1990 and Kuss, 1994]. Therefore, placental estrogen synthesis in them depends on a source of androgen precursors from the fetus' steroidogenic tissues (e.g., the fetal

adrenal glands and the gonads). However, the trophoblast cells in some species (e.g., rat, pig, sheep, and cow) express 17α- hydroxylase [Conley et al., 1992; Knight, 1994; Conley et al., 1994; Johnson, 1992; Mason et al., 1989; and Durkee et al., 1992] and can synthesize androgens. Thus, the expression of StAR gene appears to be limited to steroidogenic tissues, which exhibit acute regulation of steroidogenesis, but not in the placenta or placenta derived cells [Sugawara et al., 1995] suggesting involvement of other factors in cholesterol delivery to the P450 system. Of note, the ability of N-218 MLN64 (a truncated MLN64 protein in which 218 amino-terminal residues are deleted, hence named N-218 MLN64, which has 37% amino acid identity with StAR and 50% of StAR's steroidogenic activity) to transport cholesterol between the membranes of artificial phospholipid vesicles indicates that no other proteins are necessary for the transport activity of N-218 MLN64.

1.4 Cholesterol—Its Importance as a Starting Substrate and Need for Cholesterol Import to Mitochondria

Cholesterol, which is an essential component of all animal cell membranes, plays a critical role in defining a membrane's biochemical and biophysical characteristics [Elustondo et al., 2017]. Notably, the cholesterol content of the plasma membrane and different subcellular organelle membranes differ substantially. For example, relative to the ER and mitochondria membranes, the cholesterol content of the plasma membrane is approximately 40-fold higher [Azhar and Reaven, 2002]. As cholesterol levels are low in the mitochondrial membranes, cholesterol must be transported to the IMM for the initiation of steroidogenesis. In addition, the OMM and IMM must be supplied with cholesterol for the membrane's biochemical and biophysical characteristics at large. Thus, the mitochondrial membranes in steroidogenic cells are distinct from non-steroidogenic cell types in relation to their dual requirements of cholesterol. First, the very low levels of cholesterol in the IMM allow to control steroidogenesis through the regulation of cholesterol transport to the IMM (including the acute regulation in response to steroidogenic stimulation), which serves as the 'committed step' in steroidogenesis and are distinct from the committed steps in other biosynthetic and metabolic pathways that are generally governed by specific enzymes. Second, because of the very low levels of cholesterol contents of the mitochondrial membranes, even small changes can have a substantial impact on the biophysical

and functional characteristics of the membrane and are likely to alert the mitochondria to changes in cholesterol content. Consistent with this notion, many studies have suggested that mitochondrial membrane cholesterol can influence mitochondrial function (independent of steroidogenesis) and may contribute to the pathology of diseases related with mitochondrial abnormalities [Montero et al., 2008; 2010]. However, our current understanding of the mechanisms involved remain unclear. As steroidogenesis is essential to life, it is not surprising that many pathways for cholesterol supply to the steroidogenic mitochondria have been described. Thus, deficiency in one pathway can be taken care of by other pathways to ensure normal steroidogenesis. However, our current understanding of cholesterol delivery from the OMM to the IMM in general and its distribution between them when cholesterol levels change substantially in particular are largely unknown. This scenario raises an obvious question about steroidogenic cells because in these cells, cholesterol serves as the substrate for the synthesis of all steroid hormones [Stocco, 2000]. To overcome such a challenging scenario, the steroidogenic cells have become highly evolved to perform cholesterol uptake, mobilization, and trafficking to the mitochondria to perform steroidogenic functions.

Cholesterol has many versatile characteristics. On the one hand, cholesterol plays a critical role in determining the biochemical and biophysical properties of cellular membranes, whereas on the other hand, cleavage of its six-carbon unit side chain from cholesterol molecule (at the beginning of enzymatic step in the steroidogenic pathway) in combination with its oxygenation at certain residues changes its chemical properties and functions [Midzak and Papadopoulos, 2016]. This change in chemical properties dramatically shifts its biological role from membrane structure-function to cell signaling and transcriptional regulation [Midzak and Papadopoulos, 2016].

Cholesterol may be produced *de novo* from acetate in steroidogenic cells through a series of enzymatic pathways mainly located in the endoplasmic reticulum (ER) [Porter and Herman, 2011]. However, circulating lipoproteins serve as a major source for most steroidogenic cholesterol. High-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) are taken up via the scavenger receptor B1 (SR-B1) and by receptor-mediated endocytosis, respectively [Miller, 2013]. In addition, current literature suggests that steroidogenic cell-extrinsic (HDL- and LDL-mediated) and cell-intrinsic (*de novo*) supply of cholesterol works in a coordinated manner and are regulated by the intracellular pool of cholesterol [Miller, 2013]. Moreover, as the enzyme P450scc system, which is required for the initiation of steroidogenesis is localized to the IMM, the transfer of cholesterol from the OMM to the IMM is an essential step for steroidogenesis. Thus, the

maintenance of the readily available intracellular cholesterol pool and its transport to steroidogenic mitochondria is central to steroidogenesis.

Cholesterol functions as the precursor substrate for the biosynthesis of all steroid hormones, which begins in the IMM by the cytochrome P450scc enzyme system leading to the formation of pregnenolone, the first steroid in the steroidogenic pathway. To ensure that this essential step proceeds normally, the steroidogenic cells have evolved a robust mechanism to maintain cholesterol pool in the cytoplasm and then traffic to the steroidogenic mitochondria when needed [Stocco, 2000]. This is accomplished through a series of coordinated steps that involve various intracellular organelles, including lysosomes and lipid droplets for cholesterol mobilization, and the StAR protein for cholesterol trafficking to the steroidogenic mitochondria [Rone et al., 2009 and Azhar, 2003]. Of note, cholesterol content of the IMM is relatively very low in comparison with that of the OMM, and in steroidogenic cells, the pool of cholesterol available for steroidogenesis in the form of lipid droplets is segregated from the mitochondrial membrane cholesterol. Understanding the mechanisms that control mitochondrial cholesterol homeostasis and trafficking pertaining to steroidogenic and non-steroidogenic functions of mitochondria may provide new insights into diseases related to mitochondrial dysfunction.

1.4.1 Heterogeneity in Cholesterol Distribution and Cellular Compartmentalization of Steroidogenesis

Cholesterol is a versatile lipid, which is synthesized by animal cells and is an integral component of their subcellular membranes [Elustondo et al., 2017]. Moreover, cholesterol is the precursor substrate for all steroid hormones. Cholesterol constitutes approximately 30–40% of total cellular lipids, and nearly 60–80% of total cellular cholesterol is present in the plasma membranes [Elustondo et al., 2017]. In addition to the plasma membranes, cholesterol concentrations are high in the Golgi apparatus. In contrast, cholesterol concentrations are very poor in the endoplasmic reticulum and mitochondrial membranes. Thus, cholesterol concentration is highly heterogeneous in cellular membranes, ranging from 0.5–1% in the SER to 50–60% in the plasma membranes, and 0.1% to 0.2% in the IMM and OMM, respectively [Elustondo et al., 2017]. In cellular membranes, cholesterol influences many biophysical and biochemical aspects of membranes and membrane protein functions. Such heterogeneity in cholesterol composition in different subcellular membranes (e.g., ER and PM) and within organelle membranes (e.g., IMM

and OMM) would imply that cholesterol distribution and homeostasis must be carefully regulated in relation to membrane-specific functions. As IMM, where steroidogenesis begins, is cholesterol-poor in comparison to other subcellular membranes and compartments, it is predictable that the delivery of cholesterol to the IMM may serve as a critical regulatory step for steroidogenesis. As expected, many studies have established that this is indeed the case. Moreover, the low basal levels of cholesterol in mitochondrial membranes make the mitochondria sensitive to changes in cholesterol content, which can have a substantial impact on various non-steroidogenic functions of the mitochondria, as IMM is considered the most protein-enriched membrane in the cell. This would require a strict regulation of cholesterol handling in steroidogenic cells to protect mitochondria and mitochondria-mediated non-steroidogenic vital cellular functions. Thus, it is possible that the growing number of mitochondrial attributes and other cellular events (i.e., autophagy, lipophagy and mitochondrial dynamics) that are linked with cholesterol mobilization and trafficking in steroidogenic cells provide some flexibility to carry on steroidogenic and non-steroidogenic functions successfully under varying conditions and meet the diverse physiological needs of different steroid hormones. Thus, a possibility exists that autophagy, lipophagy and mitochondrial dynamics that have been implicated in supporting steroidogenesis protect the steroidogenic mitochondria from the potentially damaging effects of cholesterol, remove damaged mitochondria, and recycle biomolecules, including the maintenance of cholesterol homeostasis. It is likely that blocking or interfering with these protective mechanisms would lead to mitochondrial dysregulation and impaired steroidogenesis. Thus, an execution of the cell type-specific function of mitochondria (e.g., steroidogenesis) would necessitate a protection of the cell-neutral functions of mitochondria in an integrated fashion. Consistent with this notion, it is important to note that these activities (i.e., autophagy, lipophagy, mitochondrial dynamics), which have been implicated in steroidogenesis, are also operative in non-steroidogenic cells with cell type-specific mitochondrial functions, such as brown adipocytes, cardiomyocytes, hepatocytes. In addition, the compartmentalization of intracellular cholesterol in lipid droplets (LDs) in steroidogenic cells may not only provide a readily available pool for steroidogenesis, but also allow its portioning from the structural membrane cholesterol, and in protecting the functional integrity of mitochondrial and other organelles. Notably, once cholesterol is imported into the mitochondria, the steroidogenic process proceeds uninterrupted. Thus, the mitochondria may be seen as a portioning point between the processes involved in maintaining the readily available pool of precursor substrate cholesterol

and the multi-enzymatic steps of steroidogenesis in the IMM and SER. This arrangement makes sense and may have evolved to protect both cell-specific and cell-neutral mitochondrial functions from the potential disruptive effects of high cholesterol levels in steroidogenic cells. For example, increased mitochondrial cholesterol levels have been reported to decrease membrane fluidity [Montero et al., 2008; Baggetto et al., 1992; Colell et al., 2003; Paradis et al., 2013 and Bosch et al., 2011], which can affect the function of mitochondrial membrane proteins [Coll et al., 2003], including different transporters for metabolites [Parlo and Coleman, 1984; Paradies et al., 1999; Paradies et al., 1992 and Dietzen and Davis, 1994]. In addition, functional changes associated with increased mitochondrial cholesterol are similar to the effect of lipotoxicity in many cell types, including increased reactive oxygen species production and a pro-oxidative environment [Fernandez et al., 2009; Ha et al., 2012; Mari et al., 2006; Lluis et al., 2003; Mei et al., 2012 and Bosch et al., 2011], increased opposition to mitochondrial membrane permeabilization, and decreased oxidative phosphorylation [Montero et al., 2008; Colell et al., 2003; Bosch et al., 2011 and Montero et al., 2010]. Thus, steroidogenesis involved coordination between different cellular compartments. As such, steroidogenesis may be conceptualized as a compartmentalized process that involves a fine coordination of events in the different cellular compartments (e.g., the cytoplasm, mitochondria, and SER) to operate the cell-specific needs of the steroidogenic mitochondria without affecting the mitochondria's cell-neutral global functions.

1.5 Autophagy and Lipophagy in Cholesterol Homeostasis and Steroidogenesis

Autophagy is an evolutionarily conserved, cellular degradative pathway that involves the systematic degradation of select cytoplasmic components and organelles [Montero et al., 2010]. During this process, the cellular material destined for degradation is captured by the autophagosome, a double phospholipid bilayer organelle, which then fuses with the lysosome to degrade internalized debris to recycled cellular materials to maintain cell homeostasis [Kim et al., 2006 and Gawriluk et al., 2014]. Subsequent studies revealed that autophagy is essential for cell survival, differentiation, and homeostasis, and plays an important role during development and its dysregulation contributes to the pathogenesis of various diseases in mammals [Montero et al., 2010, Kim et al., 2006; Gawriluk et al., 2014 and Klionsky, 2000]. Over the last 10 years, autophagy (and the key mechanisms involved) have extensively been studied in relation to

metabolism and mitochondrial biology in different cell and tissue types, as well as in energy homeostasis and systemic metabolism [White et al., 2015; Kimmelman and White, 2017; Kim and Lee, 2014; Ueno and Komatsu, 2017 and Galluzzi et al., 2014]. In general, basal autophagy appears to provide protein and organelle quality control by eliminating damaged cellular components whereas starvation-induced autophagy recycles intracellular components into metabolic pathways to sustain mitochondrial metabolic function and energy homeostasis [White et al., 2015 and Kimmelman and White, 2017]. Many excellent reviews on autophagy and metabolism have been published recently [White et al., 2015; Kimmelman and White, 2017; Kim and Lee, 2014; Ueno and Komatsu, 2017 and Galluzzi et al., 2014] and therefore, will not be repeated here. Rather, we will focus on emerging evidence suggesting the importance of autophagy and its related events in cholesterol homeostasis pertaining to steroidogenesis. In steroidogenic cells, the evidence of autophagy can be traced back to 1968, when Frank and Christensen reported possible autophagic vacuoles in the interstitial cells of Guinea pig testis [Frank and Christensen, 1968]. Subsequent studies reported the formation of autophagosomes containing mitochondria and SER [Tang et al., 1988 and Yi and Tang, 1999]. The first report of a linkage between autophagy and testosterone production was reported in the context of late-onset hypogonadism, linking subnormal Leydig cell function with decreased autophagic activity [Li et al., 2011]. The authors showed that the treatment of Leydig cells with an autophagy blocker, inhibited LH-stimulated StAR protein expression and decreased testosterone production, whereas treatment with an autophagy activator, enhanced LH-induced steroidogenesis. More recently, autophagy and lipophagy (i.e., the autophagic degradation of lipid droplets) have been recognized as key processes in regulating cholesterol homeostasis and its transport to the mitochondria, as well as in the maintenance of testosterone production [Ma et al., 2018; Khawar et al., 2021 and Gao et al., 2018]. For example, Ma et al. [2018] and Khawar et al. [2021] showed that autophagy and lipophagy occur in Leydig cells in response to steroidogenic stimulation, suggesting that they play a key role in cholesterol trafficking and testosterone production. Moreover, a Leydig cell-specific disruption of autophagy was found to reduce testosterone production [Gao et al., 2018].

Lipophagy is a subtype of autophagy where the mobilization of lipids from lipid droplets are intimately linked with autophagy to deliver contents of lipid droplets to lysosomes [Ma et al., 2018]. Lipophagy has emerged as an important regulator of lipid homeostasis in different cell types. In steroidogenic cells, the utilization of cholesterol containing lipid droplets, is important

for steroidogenic cells to produce different steroid hormones, which have a wide-ranging systemic effect on sexual development and immunity, inflammation, and metabolism [Gawriluk et al., 2014]. Interestingly, the inhibition of autophagy was found to cause a decrease in lipid droplets, TGs, and cholesterol in both Leydig and adrenocortical cells [Gao et al., 2018], suggesting that autophagy plays an important role in lipid homeostasis in both steroidogenic cell types. This would imply that cell-intrinsic factors or events may regulate the dynamics of lipid droplets in steroidogenic cells (Figure1.3).

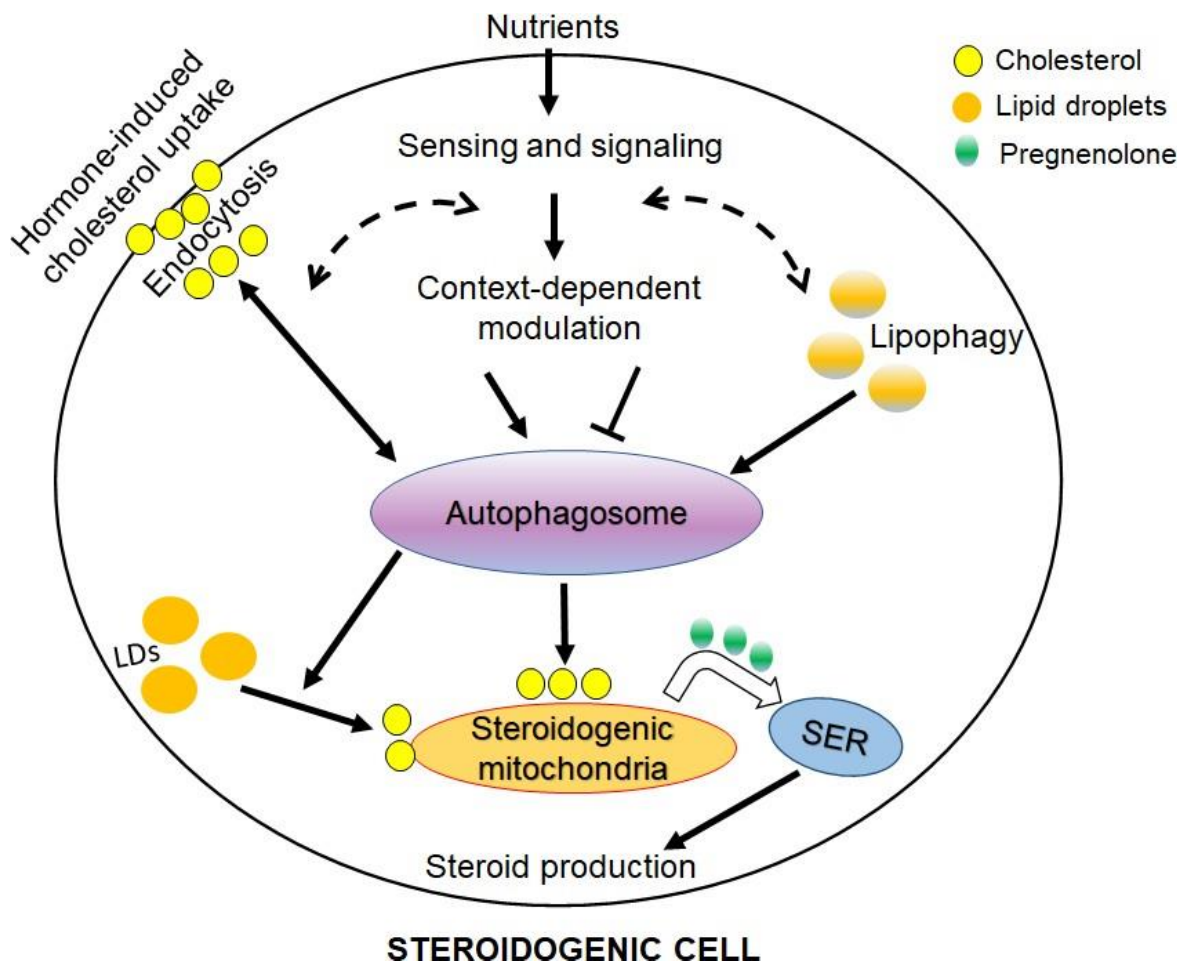


Figure 1.3. Schematic diagram depicting known (solid arrow) and potential (dashed arrow) interplay between hormone- and metabolic status-induced autophagy/lipophagy in a steroidogenic cell. It is anticipated that the interplay between these events will vary in relation to acute and chronic steroidogenesis in different steroidogenic cell types. LDs—lipid droplets; SER—smooth endoplasmic reticulum.

Furthermore, the knockdown of Beclin-1 (a crucial autophagy gene, which is the mammalian ortholog of yeast Atg6) was found to decrease LH-stimulated StAR expression and testosterone production in mouse Leydig cells, leading to the conclusion that autophagy plays a role in the maintenance of steroidogenesis in Leydig cells [Li et al., 2011]. The decline in testosterone was found to be caused by a defect in cholesterol uptake in autophagy-deficient Leydig cells [Li et al., 2011]. Further investigations revealed that disruption of autophagic flux leads to the downregulation of the SR-BI receptors leading to insufficient cholesterol supply. Notably, in both studies, the disruption of autophagy by pharmacological or genetic approaches led to a decrease in LH-stimulated StAR expression, suggesting a link between LH-induced signaling events and autophagy in the regulation of testosterone production, which may involve cholesterol trafficking to the mitochondria, as the StAR protein plays a central role therein.

In addition to the Leydig cells and adrenocortical cells, a positive effect of autophagy has been reported in porcine granulosa cell steroidogenesis in response to FSH [Gao et al., 2016]. Mechanistically, it has been shown that FSH inhibits the activation of nuclear factor-κB, which in turn leads to the activation of Janus kinase, and consequently promotes autophagy and steroidogenesis [Gao et al., 2016], providing new insights in the regulation and function of autophagy in mammalian follicle development. Moreover, similar to Leydig cells, a disruption of autophagy by Beclin-1 deletion in ovarian luteal cells of mice was found to decrease LDs and progesterone production leading to preterm labor [Gao et al, 2018]. In aggregate, a consistent finding of the disruption of autophagy by different experimental approaches in adrenal and gonadal steroidogenic cell types suggest that autophagy and related lipophagy play a crucial role in the regulation of steroidogenesis. To the best of our knowledge, such a role of autophagy in placental steroidogenesis has not been explored yet, which warrants further investigation. In addition, it would be interesting to know whether mitophagy (selective mitochondrial autophagy) plays a role in steroidogenesis, as mitophagy has been implicated in many cell types with cell-specific mitochondrial functions. Of note, estrus cycle-related changes in steroid hormones have been implicated in selective autophagy, lipophagy and mitophagy [Garcia et al., 2019]. Thus, the relationship between autophagy/lipophagy/mitophagy and steroid hormones appear to be much more complex than currently known.

In summary, these findings have shown the importance of autophagy and lipophagy in lipid regulation and steroid production. Future experiments should explore the relative importance of

autophagy and select autophagy (e.g., lipophagy and mitophagy) in the basal, acute, and chronic regulation of steroid production in different steroidogenic cell types. The elucidation of these functions will be important to understand how cell-extrinsic and cell-intrinsic factors and processes coordinate to maintain various states of steroidogenesis across steroidogenic cells, which vary substantially.

Of note, most of the work on autophagy and lipophagy has been reported in relation to Leydig cell steroidogenesis [Ma et al., 2018; Khawar et al., 2021 and Gao et al., 2018], with only a few reports focusing on adrenocortical cells [Gao et al., 2018] and ovarian granulosa cells [Gawriluk et al., 2014 and Gao et al., 2016], and with virtually none that is focused on placental cells. Thus, the findings from one steroidogenic cell type may not be generalized to all steroidogenic cells as the physiological demands of steroidogenesis vary substantially between different steroidogenic cell types, which may necessitate cell type-specific intrinsic differences. Thus, it is important to understand the context-dependent role of autophagy/lipophagy in basal, acute, and chronic steroidogenesis in different steroidogenic cell types.

1.5.1 Autophagy in Steroidogenesis—A Conserved Mechanism?

Autophagy is an evolutionarily conserved process in cell physiology, from organisms such as yeasts to mammals, which raises the question of whether autophagy's steroidogenic role, which has been reported in many mammalian species, is also involved in other species. Recently, Texada et al. [2019] showed that autophagy plays a role in the mobilization of stored precursor cholesterol and its subsequent trafficking in relation to ecdysone production in Drosophila. It was found that autophagosomes gather and transport cholesterol substrate for steroidogenesis. Thus, the results from the study by Texada et al. [2019] suggest that autophagy controls the steroidogenic process by rallying LD-derived cholesterol to supply the precursor substrate for steroidogenesis, indicating a link between new evidence related to autophagy and a well-established event involved in maintaining cholesterol homeostasis in steroidogenic cells (Figure 1.2). The interaction of the autophagosome-mediated cholesterol-trafficking with the endosome and lysosome system supports the idea of this role, since the endocytic trafficking of cholesterol is a delivery route for steroidogenesis [Saftig and Klumperman, 2009]. Thus, the cell-intrinsic events in steroidogenic cells that convert cholesterol and its intermediates into steroids might be a conserved mechanism, which requires further investigations.

1.6. Prohibitin1—A Putative Novel Player in the Steroidogenic Mitochondria and Cells at Large

Mitochondria are emerging as cellular-signaling platforms deeply integrated into diverse cellular processes. Prohibitin-1 (PHB1) is a hallmark protein of the IMM, which is involved in mitochondrial biogenesis and modulates mitochondrial dynamics [Richter-Dennerlein et al., 2014]. PHB1 and its homologous protein PHB2 form large protein and lipid scaffolds (a combination of attributes that may have implications in steroidogenesis) in the IMM that are required for structural and functional integrity of the mitochondria [Osman et al., 2009] (Figure1.4).

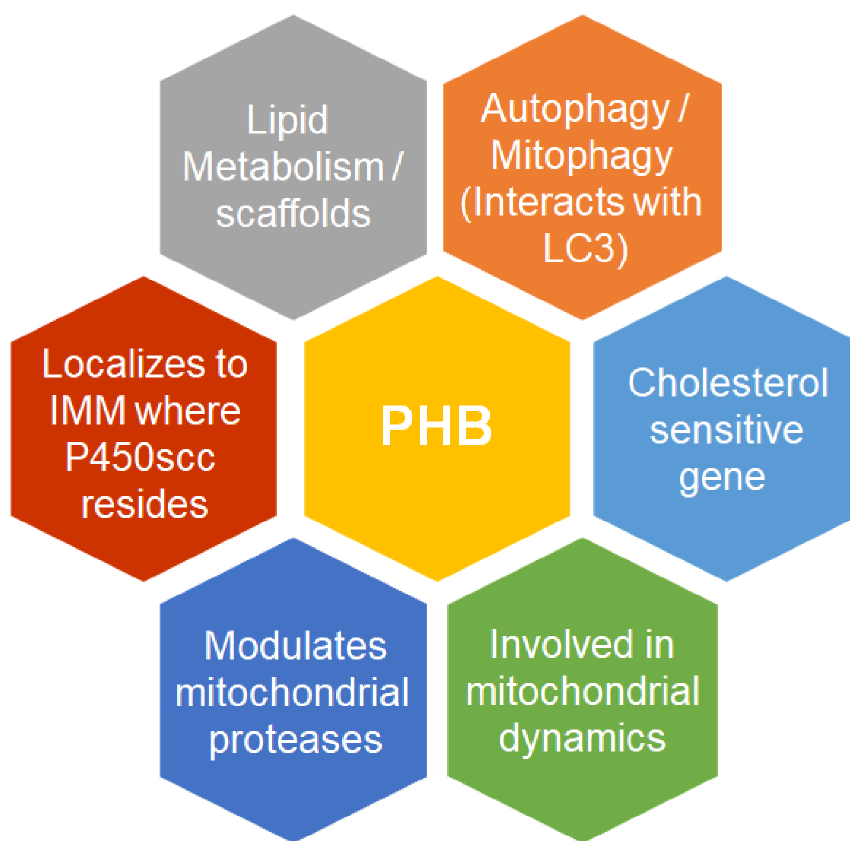


Figure 1.4. Schematic diagram depicting mitochondria, autophagy, and lipid metabolism/scaffold-related known attributes of PHB1 that makes it an apt candidate in integrating the steroidogenic mitochondria with cholesterol mobilization and trafficking in steroidogenic cells.

Both PHBs belong to a group of protein families, which are thought to function as lipid and protein scaffolds in the IMM that affect the lateral distribution of the membrane lipid and protein components [Christie et al., 2011 and Osman et al., 2009]. PHBs form hetero-oligomeric mega complexes composed of multiple PHB1 and PHB2 subunits [Tatsuta et al., 2005]. In

mitochondria, the PHB complex interacts with the m-AAA and other proteases, which act as a quality control enzyme with important regulatory functions in the IMM [Steglich et al., 1999]. Moreover, the PHB family member protein SLP2 anchors a proteolytic hub in mitochondria containing PARL and the i-AAA protease YME1L [Anand et al., 2014 and Wai et al., 2016], which are known to play a role in mitochondrial dynamics and autophagy/mitophagy. Thus, PHBs may affect mitochondrial activity in steroidogenic cells by modulating the turnover of a short-lived regulatory protein by the m-AAA protease, such as the acute regulation of StAR during steroidogenesis. In addition, PHBs may play a role in the regulation of autophagy and lipophagy because both proteins contain LC3 binding motifs and interact with each other [Wei et al., 2017], and are highly expressed in steroidogenic cells/tissues (The Human Protein Atlas).

Cholesterol serves as the metabolic precursor of all steroid hormones, and as such, steroidogenic cells and tissues can be seen as highly specialized lipid-processing cells and tissues. Since PHB1 has been implicated in lipid metabolism and homeostasis across species, including in mitochondrial phospholipids, and in autophagy/mitochondrial proteases, we speculate that PHB1 might be involved in steroid biosynthesis via lipid/cholesterol homeostasis across steroidogenic cells/tissue types. During autophagy, LC3-I is conjugated into phosphatidylethanolamine (PE) to form the LC3-PE conjugate, which is then tightly bound to the autophagosomal membranes [Tanida et al., 2008]. Notably, PHBs has a relationship with both as it serves as a binding site for LC3 and is involved in PE synthesis.

PHBs not only interacts with LC3 but is also associated with the biology of mitochondrial phospholipids, including PE [Ande et al., 2016]. It is tempting to conclude that these findings related to the autophagic regulation of factors important for cholesterol uptake and utilization in one steroidogenic cell type could be relevant for a functional interpretation across all steroidogenic cell types. However, it is likely that substantial differences between cell types are expected to exist, because the physiological demands of steroid hormones vary substantially, which are apparent in differences in their structure.

1.6.1 The Relationship between PHB Family Proteins and Cholesterol

In addition to mitochondrial biology and autophagy, the PHB family member proteins Erlin-1 and Erlin-2 are shown to be highly enriched in the detergent-soluble, buoyant fraction of sucrose gradients in a cholesterol-dependent manner [Browman et al., 2006]. However, unlike

other PHB family members (which localize to the mitochondria), these two proteins are localized to the ER. In addition to membrane localization, a common feature reported on the PHB family of proteins is that they undergo post-translational modification by palmitoylation, which is a process located in proximity of membrane targeting sequences [Huber et al., 2006]. Moreover, in a separate study [Dong et al., 2010], it was found that PHB1 is a cholesterol-sensitive gene, and its expression levels increase when cholesterol levels are low. In addition, the authors showed that the prohibitin gene promoter contains regulatory elements that respond to cholesterol insufficiency [Dong et al., 2010].

Moreover, the PHB family member protein mechanosensory protein2 (MEC-2) and Podocin have both been found to bind cholesterol to regulate the activity of associated ion channels [Huber et al., 2006]. This binding requires the PHB domain, including conserved palmitoylation sites within it and a part of the N-terminal hydrophobic domain that attaches the proteins to the cytosolic side of the plasma membrane [Huber et al., 2006]. By binding to MEC-2 and Podocin, cholesterol associates with ion- channel complexes to which these proteins bind [Huber et al., 2006]. Thus, MEC-2, Podocin, and likely many other PHB-domain proteins regulate the formation and function of large protein– cholesterol supercomplexes in the plasma membrane by forming a multimeric complex among themselves, cholesterol, and different target proteins. Moreover, in mitochondria, PHBs are anchored to the IMM, and forms complexes with the group of proteases known as ATPases (m-AAA), which are associated with diverse cellular activities.

Furthermore, PHB1 has been reported to play a role in granulosa cells [Chowdhury et al., 2015]. However, the focus of these studies was on granulosa cell proliferation, differentiation, survival, and apoptosis rather than steroidogenesis [Chowdhury et al., 2007; Chowdhury et al., 2016 and Chowdhury et al., 2013] because of our existing knowledge of PHB1's context-dependent role in cell proliferation, survival, and apoptosis in different cell types. For example, Choudhury et al. [Chowdhury et al., 2016] reported that the administration of equine chorionic gonadotropin (eCG) increases PHB1 expression in ovarian follicles and GC, but not in theca-interstitial cells within the pre-antral follicles. This increased expression of PHB1 corresponded with follicular growth and decreased after the ovulatory luteinizing hormone (LH) surge and during follicular atresia. This finding would imply that the LH surge during the ovarian cycle may negatively regulate PHB1 expression. Moreover, a change in the phosphorylation levels of PHB and increased trafficking to the mitochondria was observed. Notably, the PHB1 phosphorylation

sites under these culture conditions in response to FSH and testosterone were the Tyr^{249}, Thr^{258} and Tyr^{259} sites [Chowdhury et al., 2016 and Rikova et al., 2007], which we reported in relation to insulin signaling and lipid binding/metabolism [Ande et al., 2009; Ande and Mishra, 2009; Ande and Mishra, 2010 and Ande et al., 2011]. Thus, a possibility exists that the phosphorylation of PHB1 may play a role in steroidogenic cells in response to hormones and growth factors (e.g., trophic hormones, insulin, IGF and EGF), which are known to stimulate steroidogenesis.

Most of the arguments on the putative role of PHB1 in steroidogenesis in this section are hypothetical and are based on previous research findings on PHB1 in mitochondrial biology and lipid metabolism. Almost none of PHB1's mitochondrial and lipid metabolism attributes have been reported in relation to steroidogenesis. However, new findings (described in Chapter 4) are suggestive of PHB1 playing an important role in autophagy/lipophagy, cholesterol homeostasis and in mitochondrial dynamics in steroidogenic cells. It is our hope that others will see many research opportunities here, and that they will carry out studies that test these ideas.

1.7. Outstanding Questions and Future Research Directions

The emergence of the role of mitochondria from the site of initiation of steroidogenesis to the regulator of cholesterol mobilization, trafficking, and homeostasis to support the body's physiological levels of steroid hormone production have provided new insights and created exciting future research directions. One such example is the putative role that PHB1 plays in integrating various aspects of steroidogenic mitochondria, because of many fitting attributes it possesses related to mitochondrial biology and lipid metabolism (Figure 1.4). However, a number of fundamental questions related to our current understanding of steroidogenesis remain unanswered (Appendix A). Emerging pieces of knowledge about steroidogenesis have created opportunities to use a fresh approach to understand these underlying questions. It is expected that unraveling the molecular understanding of factors that finely tune steroid hormone production and avoid hormone insufficiency or excess may lead to the development of new therapeutic opportunities for the treatment of various diseases associated with their dysregulation.

Appendix A. Outstanding Questions

Q1. Why is steroidogenesis compartmentalized to membranes that are poor in cholesterol content (i.e., IMM and SER), but not in the PM, which is rich in cholesterol?

Q2. Why are steroidogenic enzymes membrane bound, unlike many other metabolic enzymes?

Q3. Do steroidogenic enzymes that are located in the SER play a role in SER functions like steroidogenic enzymes present in the IMM in mitochondrial function?

Q4. What is the relative importance of autophagy and lipophagy in fulfilling steroidogenic cholesterol requirements under situations of cholesterol sufficiency and insufficiency?

Q5. Why the cell's StAR level is acutely regulated in response to pituitary tropic hormones? What is the role of mitochondria in the regulation of StAR turnover?

Q6. Does PHB play a role in the functional coupling of StAR and P450scc, acute regulation of StAR and in the localization of steroidogenic enzymes in the IMM?

Q7. How do autophagy, lipophagy and mitochondrial dynamics operate under cholesterol-deficient and -sufficient states?

Q8. Does mitophagy play a role in steroidogenesis?

Q9. What is the relative importance of autophagy and lipophagy in basal, acute, and chronic steroidogenesis in different steroidogenic cell types?

Q10. What are the factors and mechanisms involved in cholesterol transport to the IMM?

Q11. Are syncytiotrophoblast mitochondria different from the mitochondria of the adrenal cortex and gonadal cells?

Q12. Do the autophagy and lipophagy processes that have been reported to play roles in steroidogenesis in gonadal cells also take part in placental steroidogenesis?

In this thesis, I have investigated some of these questions related to the potential role of PHB1 and the intracellular cholesterol pool which are described in the Chapter 4 and Chapter 5.

CHAPTER 2. STUDY RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 Study Rationale

Abundant cholesterol containing intracellular lipid droplets (LDs) and mitochondria are two distinctive features of steroidogenic cells and both are steroidogenically linked with each other. Cholesterol serves as the precursor substrate for the biosynthesis of steroid hormones whereas mitochondria represent the site for the initiation of steroidogenesis. Despite steroidogenesis has been studied for over six decades, a number of fundamental questions pertaining to trophic hormone-induced and basal steroidogenesis remain unclear. For example, transport of cholesterol across mitochondrial membrane, which is an essential step in trophic hormone-induced steroidogenesis is still elusive. Moreover, the role of cholesterol itself in steroidogenesis (beyond being a mere substrate) is not explored despite the fact that the entire framework of steroidogenesis is built around cholesterol, including its cellular uptake, intracellular storage, subsequent mobilization, transport to mitochondria and conversion to pregnenolone by P450scc enzyme. Prohibitin-1 (PHB1) is an evolutionarily conserved ubiquitously expressed protein that primarily localizes at the inner mitochondrial membrane (IMM — the site, where steroidogenesis begins), where it functions as a lipid and protein chaperone. Recently, our laboratory has developed two transgenic mouse models overexpressing PHB1 and m-PHB1 in adipocytes from the *Fabp4* gene promoter. Unexpectedly, the male m-PHB1 mice displayed high serum testosterone levels independent of LH levels, which led to the speculation of a plausible overexpression of PHB1/mPHB1 in Leydig cells and consequently altered steroid production, leading to the present investigation into the role of PHB1 in testosterone production. In addition, I set to explore the role of intracellular cholesterol pool in the regulation of cell intrinsic factors and events pertaining to steroidogenesis. Both investigations fit in the broader picture of the interplay between PHB1's mitochondrial attributes, including lipid homeostasis, and mitochondria in cholesterol handling/homeostasis and steroidogenesis.

2.2 Hypothesis

I hypothesize that PHB1 plays a role in Leydig cell steroidogenesis. In addition, I propose that the intracellular cholesterol pool is more than a precursor substrate for steroid hormones and plays a role in steroidogenesis.

2.3 Objectives

To address the hypothesis, I have the following four objectives:

2.3.1 Characterize testicular phenotype in PHB1 transgenic mice and explore the role of PHB1 in Leydig cell biology pertaining steroidogenesis.

The focus of the first objective is to characterize the testicular phenotype of PHB1 and m-PHB1 transgenic mice and illuminate the mechanisms pertaining to increased testosterone levels in m-PHB1 mice, as well as in vitro using a loss and gain of function approach in MA-10 cell (a model Leydig cell line) steroidogenesis.

2.3.2 Identify PHB's interacting partners in Leydig cell mitochondria

Here, the focus is to examine the interaction of PHB1 with key mitochondrial players of Leydig cell steroidogenesis.

2.3.3 Examine the role of PHB1 in cell signaling pathways in Leydig cells

Here, the objective is to examine the role of PHB1 in PKA and ERK signaling pathways in Leydig cells because of their crucial role in mediating steroidogenic signals.

2.3.4 Investigate the role of intracellular cholesterol pool in steroidogenic cells

The final objective is to explore the role of intracellular cholesterol pool in steroidogenesis in different steroidogenic cell types with focus on steroidogenic markers and events, including autophagy and mitochondrial dynamics in steroid production.

The first three objectives (i.e., 2.3.1, 2.3.2, and 2.3.3) pertaining to the investigation of the role of PHB1 in Ledig cell steroidogenesis are pursued in the Chapter 4 and the last objective (i.e., 2.3.4) related to a potential role of the intracellular cholesterol pool in steroidogenesis in different sterodogenic cell types is explored in the Chapter 5.

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents:

Table 3.1. Key resource table

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Atg7	Cell Signaling Technology	8558S
Anti-Cyp11A1 (P450scc)	Cell Signaling Technology	14217S
Anti-Cyp21A1	Aviva Systems Biology	OACD02918
Anti-Drp1	Cell Signaling Technology	8570S
Anti-FABP4	Cell Signaling Technology	2120S
Anti-HSL	Cell Signaling Technology	18381S
Anti-LC3	Cell Signaling Technology	12741S
Anti-Mfn2	Cell Signaling Technology	9482S
Anti-Mouse IgG HRP conjuagte	Cell Signaling Technology	7076P2
Anti-Opa1	Cell Signaling Technology	80471S
Anti-PHB1	Cell Signaling Technology	2426S
Anti-PHB2	Cell Signaling Technology	14085S
Anti-Phospho ERK1/2	Cell Signaling Technology	4695S
Anti-Phospho PKA	Cell Signaling Technology	5661S
Anti-Rabbit IgG HRP conjugate	Cell Signaling Technology	7074S
Anti-SR-BI	Abcam	ab52629
Anti-StAR	Cell Signaling Technology	8449S
Anti-Total ERK1/2	Cell Signaling Technology	9102S
Anti-Total PKA	Cell Signaling Technology	4782S
Anti-Tubulin	Cell Signaling Technology	2128S

Bacterial and virus strains

PHB1	Origene Technologies	Ande et al., 2012
PHB1shRNA	Dharmacon Inc.	RMM4431-200358818
PHB2shRNA	Dharmacon Inc.	RMM4431-200353034
StARshRNA	Dharmacon Inc.	RMM4431-200393720
XL-5	Origene Technologies	Ande et al., 2012
Y114FmPHB1	Origene Technologies	Ande et al., 2012

Chemicals, peptides, and recombinant proteins

10X RIPA buffer	Cell Signaling Technology	9806S
Cholesterol beads	Echelon Biosciences	P-BCHL
Collagenase D	Roche	11088858001
Dibutyryl cyclic AMP	Sigma-Aldrich	D0627
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	D8418-50ML
DMEM/F-12	Thermo Fischer Scientific	11330057
Dynabeads Protein G	Invitrogen	10003D
Fetal bovine serum	Thermo Fischer Scientific	A31607
hCG	Sigma-Aldrich	C8554
Horse serum	Thermo Fischer Scientific	16050130
Opti-MEM media	Thermo Fischer Scientific	11058
PBS	Sigma-Aldrich	P5368
Pen Strep	Thermo Fischer Scientific	15140
Plasmid Isolation kit	Bio-Rad	7326120
Trypsin	Thermo Fischer Scientific	25200
X-tremeGENE HP DNA transfection reagent	Roche	06366236001

Critical commercial assays

Amplex Red Cholesterol Assay kit	Thermo Fischer Scientific	A12216
FSH ELISA kit	DRG International Inc.	EIA-1288

LH ELISA kit	DRG International Inc.	EIA-1289R
Prognenolone ELISA kit	Diagnostic Biochem Canada Inc.	CAN-PRE-4500
Progesterone ELISA kit	ENZO Lifesciences	ADI-900-011
Proteome profiler human phosphor-kinase array kit	R&D Systems	ARY003B
Testosterone ELISA kit	DRG International Inc.	EIA-1559

Experimental Models: Cell lines

MA-10 cell line	Generously provided by Dr. Zhenmin Lei, University of Louisville HSC, KY	
CRISPR/Cas9-Phb-MA-10 cells	Synthego, CA	

Experimental Models: Organisms/strains

Mouse: PHB1-Tg	Developed in-house	Ande et al., 2014
Mouse: Mutant PHB1-Tg	Developed in-house	Ande et al., 2016

Software

Biorender	https://biorender.com/	Biorender
BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins	NCBI
GraphPad PRISM	https://www.graphpad.com	GraphPad software
Image Lab	https://www.biorad.com/en-ca/product/image-lab-software	ChemiDoc system BioRad Laboratories
ImageJ	https://imagej.nih.gov/ij/	

3.1.2 Media for bacterial culture

1. *Luria-Bertani (LB) media:* 10g/l Tryptone, 10g/l NaCl, 5g/l Yeast extract.

2. *LB Agar-Ampicillin plates:* 15g of agar was dissolved in 1lt of LB media and sterilized. Uponcooling, 100µg/ml ampicillin was added to LB-agar media. Next, the media was poured into100mm petri dishes and allowed to solidify in sterile conditions.

3.1.3 Buffers

1. *Phosphate buffered saline (PBS):* 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO4, 1.8mM KH_2PO4 (pH 7.6)
2. *PBST:* PBS + 0.05% Tween20
3. *SDS-PAGE running buffer:* 25mM Tris-base, 192mM Glycine, 0.1% SDS (pH 8.3)
4. *Transfer (TB) buffer*: 25mM Tris-base, 192mM Glycine, 20% Methanol
5. *Tris-buffered saline (TBS):* 20mM Tris-base, 150mM NaCl (pH 7.6)
6. *TBST:* TBS + 0.05% Tween20
7. *Tissue lysis buffer:* 100mM Tris-base, 200mM NaCl, 10% Glycerol, 1% SDS, 5mM EDTA with 1X protease inhibitor and 1X phosphatase inhibitor cocktails.
8. *Cell lysis buffer (Immunoblot analysis):* 50mM Tris-base, 150mM NaCl, 10% Glycerol, 1% SDS with 1X protease inhibitor and 1X phosphatase inhibitor cocktails.
9. *Immuoprecipitation lysis buffer:* 25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol.
10. *Sample loading buffer (2X Laemmli buffer):* 125mM Tris-base, 10% 2-Mercaptoethanol, 20% Glycerol, 4% SDS, 0.004% Bromophenol blue (pH 6.8).

3.2 Methods

3.2.1 Animal models

The development and phenotypic characterization of the PHB1 and mPHB1 transgenic mice have been described previously [Ande et al., 2014, 2016a, 2016b]. The male PHB1, mPHB1, and wild-type control mice were housed under a 12-hour light-dark cycle at 22°C and were provided with normal chow (LabDiet, St. Louis, MO) and water *ad libitum*. All procedures were

approved by the Animal Care and Use Committee of the University of Manitoba, Winnipeg, Canada (Protocol Approval #16-005, #20-008).

3.2.2 Testis retrieval

First, the mice were anesthetized with 3% isoflurane and blood was collected from the saphenous vein for hormonal analyses. Subsequently, the mice were euthanized by CO_2 inhalation and the serum was stored at –20°C until analyzed. The abdomen area was sterilized with 70% alcohol and then a peritoneal incision was made using a sharp scalpel. The skin was opened anterior to the genitals to remove each of the testicles.

3.2.3 Primary Leydig cell (LC) isolation and culture

The testes were removed from the mice, sterilized using pre-chilled 70% ethanol (two washes), and washed three times with pre-chilled PBS. Epididymis, fat, and other connective tissues were removed from the testis samples using small scissors and forceps. The tunica albuginea was then dissected, and the testis samples were then placed into a 15ml centrifuge tube containing a Dulbecco's Modified Eagle Medium/nutrient mixture F-12 (DMEM/F-12) and 0.02% Collagenase D under constant agitation (90rpm) for 30min at 37°C, followed by an incubation undisturbed at room temperature for 10min [Yamashita et al., 2011]. Subsequently, the supernatant was filtered into a fresh 15ml centrifuge tube containing 5ml fresh DMEM/F-12 medium and was centrifuged at 200 x g for 4min at room temperature. The pellet was washed twice with the fresh medium, and finally, the cells were resuspended in the DMEM/F-12 medium supplemented with 10% FBS for seeding [Yamashita et al., 2011]. The cells were kept at 37°C with 5% CO2 in a humidified atmosphere.

3.2.4 Histological analysis

Testis samples from the 4-month-old PHB1, mPHB1, and wild-type mice were fixed in 4% buffered formaldehyde solution, dehydrated, and embedded in a paraffin block. Sections were stained with hematoxylin-eosin [Ande et al., 2014, 2016a, 2016b]. The sections were analyzed under a light microscope and photomicrographs were captured using Evos (XL Core AMEX 1000, Invitrogen).

3.2.5 Transmission electron microscopy (TEM)

The transmission electron microscopy (TEM) of the testis was performed using a Philips CM10 at 80kV at the Histomorphology & Ultrastructural Imaging Platform, in the Faculty of Health Sciences in the University of Manitoba. In brief, the testis samples were excised into small pieces (< 1mm^3) and fixed with 3% glutaraldehyde in 0.1M Sorensen's buffer for 3hours. After fixation, the cells were resuspended in 5% sucrose in 0.1M Sorensen's buffer and then embedded in EPON^{TM} resin. TEM analysis was performed on ultra-thin sections (100nm) and stained with uranyl acetate and counterstained with lead citrate [Ande et al., 2014].

3.2.6 Plasmid DNA transformation of competent *E.coli* cells

The competent *E. coli* cells (Cat # C2987H) were purchased from New England Biolabs (NEB). 5-α competent *E. coli* cells were thawed on ice for 10min. 20ng of wild-type PHB1 and mutant PHB1 (Y114F) plasmid DNA was added to the competent cells separately, and the mixture was placed on ice for 30min. After 30min, cells were heat shocked at 42°C for 30sec. Subsequently, cells were placed on ice for 5min, cells were then added to 1ml of LB media and were grown at 37°C for 1h. Then, 0.1ml of saturated culture was inoculated in 100ml of LB media in a 500ml flask and allowed to grow at 37°C under constant shaking (200-250rpm) for 18h, until an A260 value of 0.6 was reached. Then the cells were centrifuged at 2500 x g for 10min at 4°C [Ande et al., 2009 and Ande and Mishra, 2009]. Glycerol stocks of shPHB1 (Cat#RMM4431-200358818) and shStAR (Cat# RMM4431-200393720) were purchased from from Horizon Discovery and were grown according to manufacturer's instructions.

3.2.7 Plasmid DNA isolation

The bacterial cultures obtained after overnight incubation were subjected to either plasmid DNA isolation or frozen as glycerol stocks for further use. The plasmid DNA was isolated from the using the QIAprep plasmid extraction kit as per the manufacturer's instructions. The A260/280 ratio value in the range of 1.7-1.8 as measured by Nanodrop spectrophotometer confirmed the purity of the isolated plasmid DNA.

3.2.8 Cell culture

Steroidogenic model human trophoblast (BeWo) cell line and adrenocortical (Y-1) cell lines were obtained from ATTC (Manassas, VA). Dr. Zhenmin Lei, University of Louisville HSC, KY generously provided MA-10, a Leydig cell line. Dr. Vernon Dolinsky (Department of Pharmacology and Therapeutics, University of Manitoba) generously provided non-steroidogenic rat H9c2 myocardial cell line. All cell culture media and related reagents were obtained from Life Technologies Inc. (Thermo Fischer, Canada), except lipoprotein depleted FBS, which was obtained from (KALEN Biochemical, Germantown, MD).

The choriocarcinoma BeWo cells were cultured in F-12K medium supplemented with 10% FBS and 1% penicillin/streptomycin and first differentiated into syncytial trophoblasts [Msheik et al., 2019] before subjected to cholesterol depletion. MA-10 cells were cultured in DMEM-F12K medium supplemented with 15% horse serum and 1% penicillin/streptomycin whereas Y-1 cells were cultured in F-12K Ham medium supplemented with 10% FBS, 5% horse serum and 1% penicillin/streptomycin [Forti et al., 2002]. H9c2 cells were cultured in DMEM medium supplemented with 10% FBS, and 1% penicillin/streptomycin. All cell lines were cultured and maintained at 37°C in a 5% CO2 and humidified atmosphere.

3.2.9 Cell treatment

Dosages of different trophic hormones used for the stimulation of different steroidogenic cells are based on information in the current literature. For example, BeWo and MA-10 cells were treated with hCG (20ng/ml) [Verma et al., 2021 and Riccetti et al., 2017] and Y-1 cells with ACTH (15nM) [Schimmer et al., 2015] for 6h, 12h and 24h time points under NC and CD culture conditions. In some experiments, cells were treated with db-cAMP (0.5mM) instead of trophic hormone, when appropriate or as indicated.

3.2.10 Depletion of intracellular cholesterol pool in steroidogenic cells

For this, each cell type was washed 3-times with PBS and then cultured in cholesterol depleted (CD) cell culture conditions (i.e., respective cell type-specific culture medium supplemented with lipoprotein depleted FBS (Cat # 880100; KALEN Biochemical, Germantown, MD) containing only 0.04mg/ml cholesterol (instead of normal FBS, which contains around

1.40mg/ml cholesterol). An approximately 35-fold reduction in cholesterol content in lipoprotein depleted FBS compared with normal FBS. Cells were kept in CD culture condition for different time points (i.e., 6h, 12h and 24h). Cells in control experimental group in each case was continuously grown under normal condition (i.e., supplemented with normal FBS or horse serum, as applicable). BeWo cells were cultured under CD condition after differentiation into syncytial trophoblasts [Msheik et al., 2019].

3.2.11 Cell transfections

In chapter 4, after 36h of culture, MA-10 cells were serum-starved for 6h followed by transfection. The pCMV6-XL5 vector containing the human PHB1 clone was purchased from Origene Technologies and the cloning of the m-PHB1 cDNA construct has been reported previously [Ande et al., 2009, 2012]. In brief, tyrosine 114 to phenylalanine (Tyr114Phe) mutant-PHB1 was made using site-directed mutagenesis kit (Stratagene, USA) following the manufacturer's instructions. The following primers were used for generating mutant-PHB (forward: 5′CAGCATCGGAGAGGACTTTGATGAGCGTGTGC 3′ and reverse: 5′ GCACACGCTCATCAAAGTCCTCTCCGATGCTG3′). Authenticity of all constructs was confirmed by DNA sequencing. Cell transfections were performed using X-tremeGene HP transfection reagent (Roche, Sigma Aldrich) according to manufacturer's protocol [Ande et al., 2009, 2012]. After 30-36h of transfection, cell lysates were prepared using 1X RIPA lysis buffer (50mM Tris–Cl, pH 7.4) containing protease inhibitors and subsequently processed for further analysis. In Chapter 5, MA-10 cells, Y-1 cells were transfected with shStAR for 36h followed by lysis using 1X RIPA lysis buffer (50mM Tris–Cl, pH 7.4) containing protease inhibitors.

3.2.12 CRISPR/Cas9-Phb-MA-10 cells

In chapter 4, CRISPR/Cas9-mediated Phb knockout MA-10 cells (CRISPR/Cas9-Phb-MA-10) was established using a custom service provided by Synthego (Menlo Park, CA). The editing efficiency after expansion of guide RNA (gRNA: UUACCAGGGACACGUCAUCC targeting Exon 5) transfected MA-10 KO cell pool was 79%. The site-specific targeting was confirmed using PCR and sequencing primers (F: GGGTTATAGCCATGAGTGTGCC and R:

GTGTGCGGCAGACGAAACCT). Subsequently, knockout pool of MA-10 cells was subjected to serial dilution as per the manufacturer's instruction and following standard protocol to establish clonal CRISPR/Cas9-Phb-MA-10 cell line.

3.2.13 MTT reduction assay

The effect of CD on cell viability was enumerated by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [Mishra et al., 2004]. For this, cells were seeded at a plating density of 3×103/well and cultured for 24h to allow them to adhere to the plate. The culture medium was then changed to CD condition and then cells were allowed to grow for another 24h (i.e., maximum time point used in CD experimental group). Cells in control group (NC condition) were continuously grown under normal culture condition (i.e., cell type-specific). At the end 10µg/100µl MTT was added, and incubation continued for 3h. Subsequently, the tetrazolium products were solubilized in acidic isopropanol and OD was read at 570nm.

3.2.14 Western blotting

At each time point, cells were washed with ice-cold PBS and lysed in RIPA cell lysis buffer supplemented with proteinase and phosphatase inhibitor cocktails [Ande et al., 2009 and Ande et al., 2012]. After lysis, cell lysates were kept at 4°C for 30min, vortexed intermittently every 5min and then centrifuged at 4°C for 10min at 13000rpm. The supernatant was transferred into a new Eppendorf tube for subsequent use. The total protein concentration for all lysates was measured by Bradford protein assay method using bovine serum albumin as a standard. Next, protein samples (20µg each) were loaded for electrophoresis on 12% mini gel by standard SDS-PAGE procedures and electro transferred to polyvinylidene difluoride (PVDF) membranes by wet transfer method [Ande et al., 2009 and Ande et al., 2012]. Then, the blots were blocked with 5% non-fat dry milk in TBS, 0.1% Tween-20 for 1h followed by incubation with respective primary antibodies overnight at 4ºC with gentle shaking. The membranes were washed in TBST for 3 x 10min and incubated with respective secondary antibody conjugated with horseradish peroxidase for 1h at room temperature. Immunoreactive proteins were detected by chemiluminescence with Western blotting luminol reagent, and the images were captured with a ChemiDoc system (Bio-Rad Laboratories).

3.2.15 Immunoprecipitation

The immunoprecipitation of PHB1, PHB2, StAR and P450scc was performed using a protein specific antibody and a Dynabeads protein G suspension according to the manufacturer's protocol. In brief, 10µL of the protein-specific antibody (as applicable) were added to 500µL of the cell lysate and incubated overnight on a rotating device at 4°C [Ande et al., 2009 and Ande and Mishra, 2009]. At the end of the incubation, 20µL of the Dynabeads protein G suspension was added to each tube and further incubated for 2h. Subsequently, the pellets were washed 5 times in ice-cold PBS, resuspended in 2X loading buffer and analyzed by immunoblotting.

3.2.16 Cholesterol binding assay

An assay of the cholesterol beads was performed according to the manufacturer's instructions. Briefly, to 500µl of cell lysate, 20µl of the cholesterol bead suspension was added and incubated at 4°C for 3h on a rotating device. Subsequently, the pellets were washed 3 times with ice-cold PBS by centrifugation at 2000rpm for 2min at room temperature. Finally, the pellets were resuspended in 2X loading buffer and analyzed by immunoblotting using the anti-PHB1 and anti-PHB2 antibody.

3.2.17 Amplex^{TM} red cholesterol assay

The cholesterol levels in BeWo, MA-10 and Y-1 cells grown under NC and CD culture conditions were measured using an enzyme-coupled Amplex^{TM} Red cholesterol assay kit as per the manufacturer's (Thermo Fischer Scientific, CA) instructions. This assay kit provides a simple fluorometric method for the sensitive quantitation of cholesterol using a fluorescence microplate reader or fluorimeter. The assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters. Cholesteryl esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield H_2O_2 and the corresponding ketone product.

3.2.18 Proteome profiler human phospho-kinase array

The MA-10 cells were transfected with PHB1 and mPHB1 plasmid DNA for 36h and treated with dibutyryl cAMP (db-cAMP) for 2h, and cell lysates were prepared post-transfection and treatment. The phosphorylation profile of the signaling pathways was detected by using the

Proteome profiler human phospho-kinase array kit with 200µg of protein samples. The assay was performed according to the manufacturer's instructions and the images were captured using the Bio-Rad imaging system. Blot densities were analyzed by quantitative densitometry and each dot intensity was normalized to the reference dot's intensities.

3.2.19 Measurement of hormone levels

Prognenolone (CAN-PRE-4500, Diagnostics Biochem Canada Inc.), Progesterone (ADI-900-011, ENZO Lifesciences), FSH (EIA-1288, DRG International Inc.), LH (EIA-1289R, DRG International Inc.), and Testosterone (EIA-1559, DRG International Inc.) levels were measured according to the manufacturer's instructions.

3.2.20 Statistical analysis

Quantification of band densities, lipid droplets, mitochondrial numbers, and lipid droplet areas were performed using ImageJ software (https://imagej.nih.gov/ij/). GraphPad Prism 6 software was used for the statistical analysis in all experiments. For comparisons between two groups, a two-tailed student's t test was performed. An anlysis of variance (ANOVA) with Dunnett test was performed to compare every mean to a control mean (e.g., time-dependent effect of hormonal or cAMP stimulation in Chapter 4) whereas Tuckey test was performed for multiple comparisons (e.g., Control (wild type or vector control as applicable), PHB1 and mPHB1 experimental groups in Chapter 4, as well as NC and CD experimental groups with/without hormonal stimulation in Chapter 5). A P value of < 0.05 was considered statistically significant in all cases. The graphs represent means, and error bars indicate the standard error of the mean (SEM). All experiments were repeated for at least 3 times or represent 6mice/experimental group and p values are reported in the respective figure legends or indicated in figures.

CHAPTER 4

The Discovery of a Regulatory Role of Prohibitin-1 in testosterone Production.

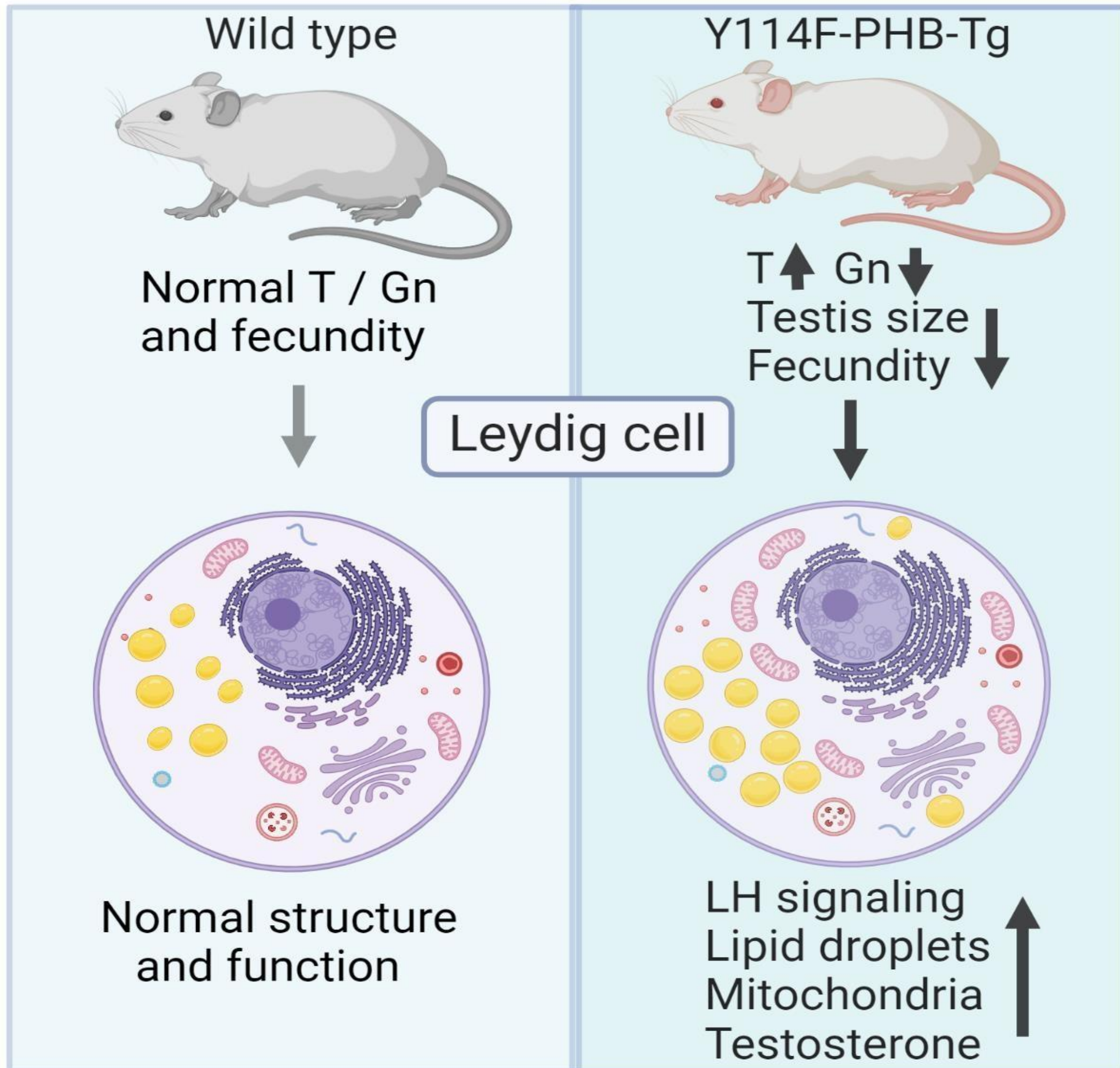
This section has been published as a research article titled:

"**Prohibitin-1 Plays a Regulatory Role in Leydig Cell Steroidogenesis**"

Geetika Bassi, Suresh Mishra

iScience, 2022 PMID:35434552 https://doi.org/10.1016/j.isci.2022.104165

Graphical abstract



Highlights

- Tyr^{114}Phe-PHB-1 transgenic male mice reveal PHB-1's role in testosterone production
- PHB-1 coordinates steroidogenic signaling and events in testosterone biosynthesis
- Tyr^{114} residue in PHB-1 plays a regulatory role in testosterone production

4.0 Summary

Mitochondria are essential for steroidogenesis. In steroidogenic cells, the initiation of steroidogenesis from cholesterol occurs on the matrix side of the inner mitochondrial membrane by the enzyme P450scc. This requires cholesterol import from the cytoplasm through the outer mitochondrial membrane, facilitated by the StAR protein. The subsequent steps leading to P450scc remain elusive. Here we report that the male transgenic mice that expressed a mutant form of a mitochondrial protein prohibitin-1 ($PHB1^{Tyr114Phe}$) from the *Fabp-4* gene promoter displayed smaller testes, higher testosterone, and lower gonadotropin levels compared with the PHB1-expressing and wild-type mice. Subsequent analysis of the testis and Leydig cells from the mice revealed that PHB1 played a previously unknown regulatory role in Leydig cell steroidogenesis. This includes a role in coordinating cell signaling, cholesterol homeostasis, and mitochondrial biology pertaining to steroidogenesis. The implications of our finding are broad as the initial stages of steroidogenesis are indistinguishable across steroidogenic cells.

4.1 Introduction

Steroid hormones are essential to life, as they regulate critical phases of development, such as puberty and reproductive ability [Wood et al., 2019]. Altered levels of steroid hormones are associated with various pathological conditions, including infertility [Reichman et al., 2017], metabolic and immune dysregulation [Faulkner et al., 2019], as well as hormone-dependent cancers [Rodriguez-Gonzalez et al., 2008]. Thus, steroid hormone biosynthesis is finely regulated to ensure adequate amounts are produced, but also to avoid hormone insufficiency or excess. In this context, the feedback relationship between the different trophic hormones and the respective steroid hormones (e.g., ACTH-glucocorticoids, LH-testosterone and LH/FSH-estradiol) is well-established [Oyola et al., 2017 and Kaprara et al., 2018]. The trophic hormone-induced cellular events in steroidogenic cells leading to steroid hormone production span different cellular compartments (i.e., the cytoplasm, mitochondria, and smooth endoplasmic reticulum) [Miller et al., 2011]. This arrangement is likely to facilitate a fine coordination between steroidogenic events in the different cellular compartments to control hormone levels within a normal physiological range. However, our knowledge of intracellular regulatory factors and mechanisms that may coordinate steroidogenic processes between different cellular compartments to maintain steroidogenic homeostasis (i.e., avoid hormone deficiency or excess) in steroidogenic cells remains limited. This knowledge is important because of the inherent relationship between the regulation of trophic hormones at the hypothalamus-pituitary level and the potential pathological consequences of their dysregulation in steroidogenic glands through altered tropic hormones, and consequently in the body at large, due to dysregulated steroidogenesis. For example, a perturbed steroidogenesis in the adrenal glands and the testis may lead to pathologies related to chronic high or low levels of trophic hormones due to a dysregulated negative feedback loop at the hypothalamus-pituitary level, such as congenital adrenal hyperplasia and hypogonadotropic hypogonadism, respectively.

The fundamental framework of steroid hormone biosynthesis across major steroidogenic tissues is very similar, especially within mitochondrial steps, which are indistinguishable [Midzak et al., 2016]. For example, cholesterol is the common substrate for all steroid hormones, and its transport by the steroidogenic acute regulatory protein (StAR) to the mitochondria, and the subsequent utilization by the cytochrome P450 side chain cleavage (P450scc, encoded by the

CYP11A1 gene) enzyme for the initiation of steroidogenesis, is a key step in all steroidogenic tissues [Monté et al., 1998]. However, the identity of the mitochondrial protein(s) that couple the StAR function at the cytoplasmic side of the outer mitochondrial membrane (OMM) with the P450scc enzyme at the matrix side of the inner mitochondrial membrane (IMM), and the mechanisms involved, remain elusive. In addition, emerging evidence suggests that autophagy and lipophagy in Leydig cells play a role in intracellular cholesterol homeostasis and in the maintenance of testosterone production [Gao et al., 2018, Ma et al., 2018]. Moreover, Leydig cell steroidogenesis is regulated by structural and functional changes in mitochondria [Park et al., 2019 and Duarte et al., 2014]. Mitochondrial fusion and fission, collectively known as mitochondrial dynamics, have been reported to play a role in steroidogenesis [Wasilewski et al., 2012 and Duarte et al., 2012]. In Leydig cells, hormonal stimulation triggers mitochondrial fusion through the upregulation of the fusion protein mitofusin 2, a process that is essential for steroid hormone production [Castillo et al., 2015]. Thus, the mitochondria are not merely a site for the initiation of steroidogenesis involving the conversion of cholesterol to pregnenolone, but also appears to play a multifaceted role in steroidogenesis, including cholesterol proportioning and in the maintenance of cholesterol and steroidogenic homeostasis [Midzak et al., 2016]. Thus, a better understanding of the interplay between different steroidogenic cell-specific functions (e.g., cholesterol homeostasis and mitochondrial attributes) is essential to advance our understanding of this fundamental biological process in Leydig cells and in other steroidogenic cell types.

Over the last 15 years, our lab has been interested in elucidating the role and regulation of an evolutionarily conserved, but so far poorly characterized protein, prohibitin-1 (PHB1) [Ande et al., 2016; Ande et al., 2012; Ande et al., 2014]. For instance, we have identified the Tyr^{114} residue in PHB1 as an important phosphorylation site in relation to membrane signaling (e.g., PI3K-Akt and MAPK-ERK) [Ande et al., 2012; Ande et al., 2009; Ande and Mishra, 2009], demonstrated its regulatory role in cell signaling, and discovered PHB1's role in adipogenesis and lipid homeostasis [Ande et al., 2012 and Ande et al., 2014], which have been further confirmed by others [Kang et al., 2013 and Kim et al., 2013]. Recently, we have shown that the transgenic mice overexpressing of PHB1 or mutant PHB1 ($PHB1^{Tyr114Phe}$ or mPHB1) from the fatty acid binding protein-4 (*Fabp4*) gene promoter (for adipocyte-specific expression) develops obesity, which is mediated through upregulating mitochondrial biogenesis in adipocytes [Ande et al., 2014 and Ande et al., 2016]. Unexpectedly, during their phenotypic characterization, we found that the

mPHB1 mice have smaller testes but higher serum testosterone levels. Further investigation revealed that the PHB1 and mPHB1 mice overexpress PHB1 in their Leydig cells, which is consistent with a recent report showing that Fabp4 is expressed in Leydig cells [O'Hara et al., 2015]. However, PHB1's role in Leydig cell steroidogenesis is virtually unknown in current literature. This prompted me to investigate the role of PHB1 in Leydig cell biology. I found that PHB1 is an important steroidogenic target gene in Leydig cells and plays a regulatory role in Leydig cell steroidogenesis, including in cell signaling, cholesterol homeostasis, and mitochondrial biology involved in steroidogenesis. This requires the Tyr^{114} residue in PHB1, and its substitution with a phenylalanine (Phe) residue leads to the upregulation of testosterone production. Thus, I discovered a previously unknown role of PHB1 in regulating interconnected steroidogenic events in different cellular compartments in Leydig cells. The implications of our findings are broad as the fundamentals of steroidogenesis, such as cholesterol homeostasis, cholesterol transport to the mitochondria, and the initiation of steroidogenesis, are common among all steroidogenic tissues.

4.2 Materials and Methods

Materials used in Chapter 4 are described in Chapter 3 under section: Chemicals and reagents key resource table. Methods used in this chapter are described in Chapter 3 under the sections: animal models (3.2.1), testis retrieval (3.2.2), primary Leydig cell (LC) isolation and culture (3.2.3), histological analysis (3.2.4), transmission electron microscopy (TEM) (3.2.5), cell culture (3.2.8), cell transfections (3.2.11), CRISPR/Cas9-Phb-MA-10 cells (3.2.12), western blotting (3.2.14), immunoprecipitation (3.2.15), cholesterol binding assay (3.2.16), amplex™ red cholesterol assay (3.2.17), proteome profiler human phospho-kinase array (3.2.18), statistical analysis (3.2.20).

4.3 Results

4.3.1 Male mPHB1 mice display smaller testes, elevated serum testosterone and lower gonadotropin levels

The immunometabolic phenotype of the PHB1 and mPHB1 mice have been described previously [Ande et al., 2016; Ande et al., 2014 and Ande et al., 2016]. During follow-up studies pertaining to these mouse models, I found a reduction in the siring ability of the male mPHB1 mice compared with age-matched, PHB1 and wild-type mice, which was significantly lower in the male mPHB1 mice by 6 months of age (Fig. 4.1A). A similar trend was also observed in the male PHB1 mice; however, the difference between PHB1 and wild-type was not significant (Fig. 4.1A). Moreover, a clear size difference in testes was observed between the male mPHB1 mice and wild-type mice, which was significantly smaller in the mPHB1 mice (~50% reduction in the testis weight) (Fig. 4.1B). A similar trend in testis size was also observed in the PHB1 mice; however, the difference was not significant when compared with the wild-type mice (Fig. 4.1B). In addition, the testis from the mPHB1 mice was also significantly smaller (~35% reduction) than the testis from the PHB1 mice (Fig. 4.1B) despite comparable body weights [Ande et al., 2014 and Ande et al., 2016]. However, I did not find such a difference in the size and weight of the secondary sex organs between the male transgenic and wild-type mice (not shown). This prompted me to examine serum testosterone levels within each mouse genotype. Surprisingly, serum testosterone levels were significantly higher in the mPHB1 mice compared with the age-matched PHB1 mice and wild-type mice (Fig. 4.1C). A significant difference in testosterone levels was also found between the mPHB1 and PHB1 mice, which was higher in the mPHB1 mice (Fig. 4.1C). To determine whether the higher testosterone levels in mPHB1 mice was due to its increased production from Leydig cells, we measured testosterone production from the primary Leydig cells isolated from each mouse genotype in response to the human chorionic gonadotropin (hCG, a surrogate for the luteinizing hormone (LH) as both bind to the same LH receptors and elicit a similar response) [Lei et al., 2001]. Consistent with serum testosterone levels, the Leydig cells from the mPHB1 mice produced significantly more testosterone compared with the Leydig cells from the PHB1 mice and wild-type mice (Fig. 4.1D). In addition, a similar trend was found in testosterone production by Leydig cells between the PHB1 and wild-type mice (Fig. 4.1D). In aggregate, this data suggests that PHB1 plays a regulatory role in testosterone production that involves the Tyr^{114}

phosphorylation site, because its substitution by a non-phosphorylable phenylalanine (Phe) residue in mPHB1 leads to increased testosterone production.

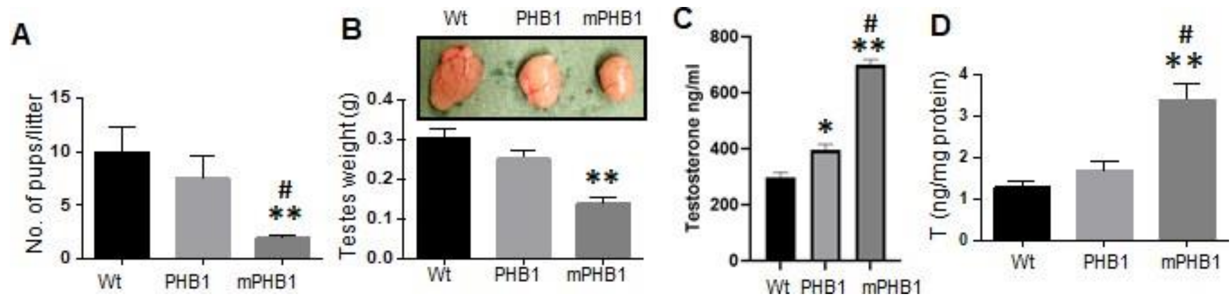


Figure 4.1. Male mPHB1 mice display reduced fertility, smaller testis, elevated testosterone and lower gonadotropin levels

(A) Histograms showing litter size at birth in wild-type (Wt) dams when sired with PHB1, mPHB1 and Wt mice separately at 6 months of age.

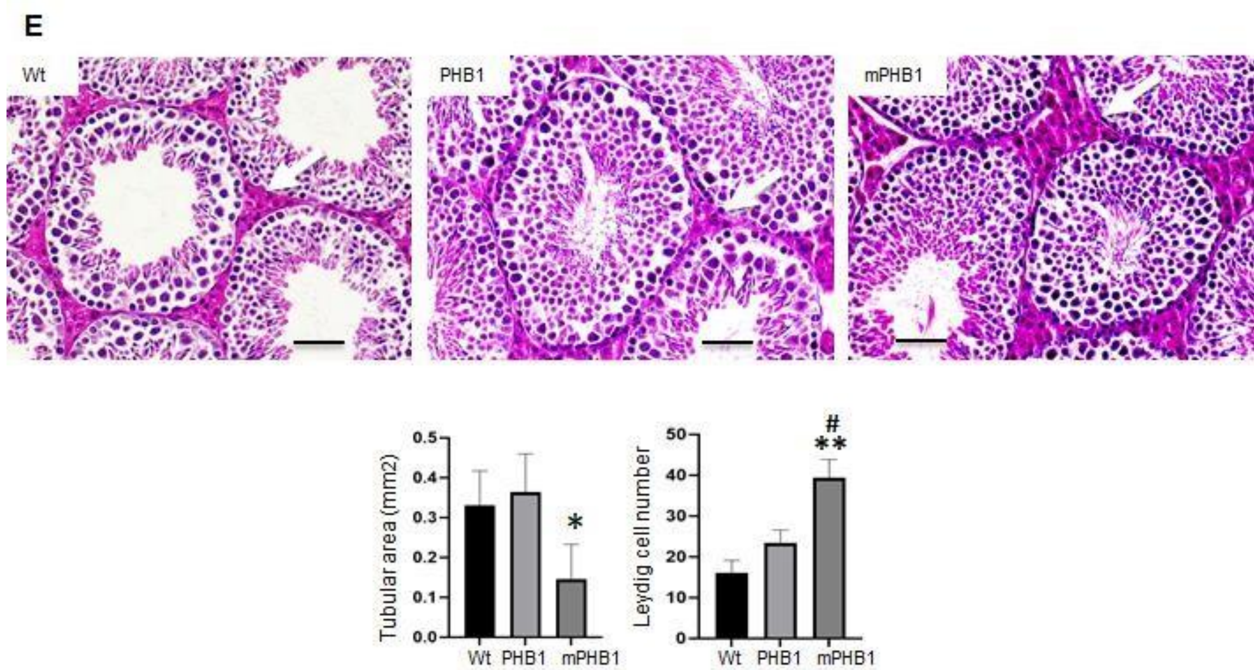
(B) Photographs showing testis dissected from PHB1, mPHB1 and Wt mice at 6 months of age (upper panel). Histograms showing quantification of testis weight from PHB1, mPHB1 and Wt mice (lower panel).

(C) Histograms showing serum testosterone levels in PHB1, mPHB1 and Wt mice at 6 months of age.

(D) Histograms showing testosterone production from primary Leydig cells derived from 4 months old PHB1, mPHB1 and Wt mice in response to hCG (20ng/ml).

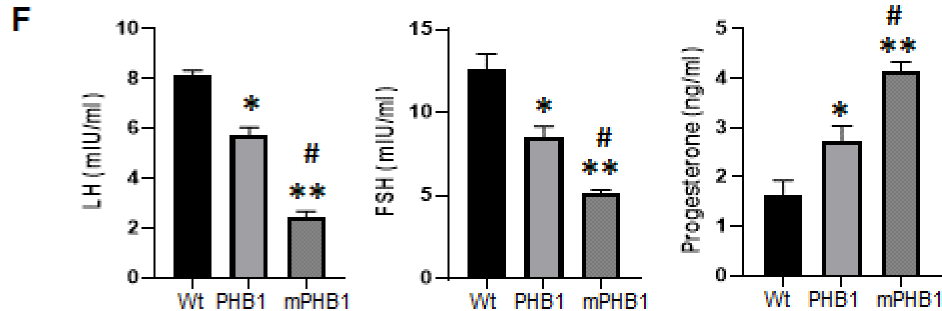
*p < 0.05, **p < 0.01 between Wt and PHB1 or mPHB1 mice, # p < 0.05 between PHB1 and mPHB1 mice. Data are presented as mean ± SEM (n = 6 mice/group or experiments repeated atleast 3 times). n.s. – not significant, Wt – wild type.

To get insight into why the differences in testis size and serum testosterone levels between the PHB1, mPHB1, and wild-type mice were occurring, we performed a histological analysis. Consistent with testis size, the area of seminiferous tubules was significantly smaller with relatively narrow lumen and reduced intra-tubular germ cell contents in mPHB1 mice compared with PHB1 and wild-type mice (Fig. 4.1E). However, the interstitial space between seminiferous tubules were relatively larger and wider in the testis from mPHB1 mice, with increased Leydig cell population (Fig. 4.1E), indicating a potential relationship between the Leydig cells and higher testosterone levels in the mPHB1 mice.



(E) Photomicrographs showing H & E-stained testis sections from PHB1, mPHB1 and Wt mice at 6 months of age. Quantification of seminiferous tubular area (right upper panel) and Leydig cell number in testicular interstitium (right lower panel) from transgenic and wild type-mice are shown by histograms. Scale bar = 20 µm.
*p < 0.05, **p < 0.01 between Wt and PHB1 or mPHB1 mice, # p < 0.05 between PHB1 and mPHB1 mice. Data are presented as mean ± SEM (n = 6 mice/group or experiments repeated atleast 3 times). n.s. – not significant, Wt – wild type.

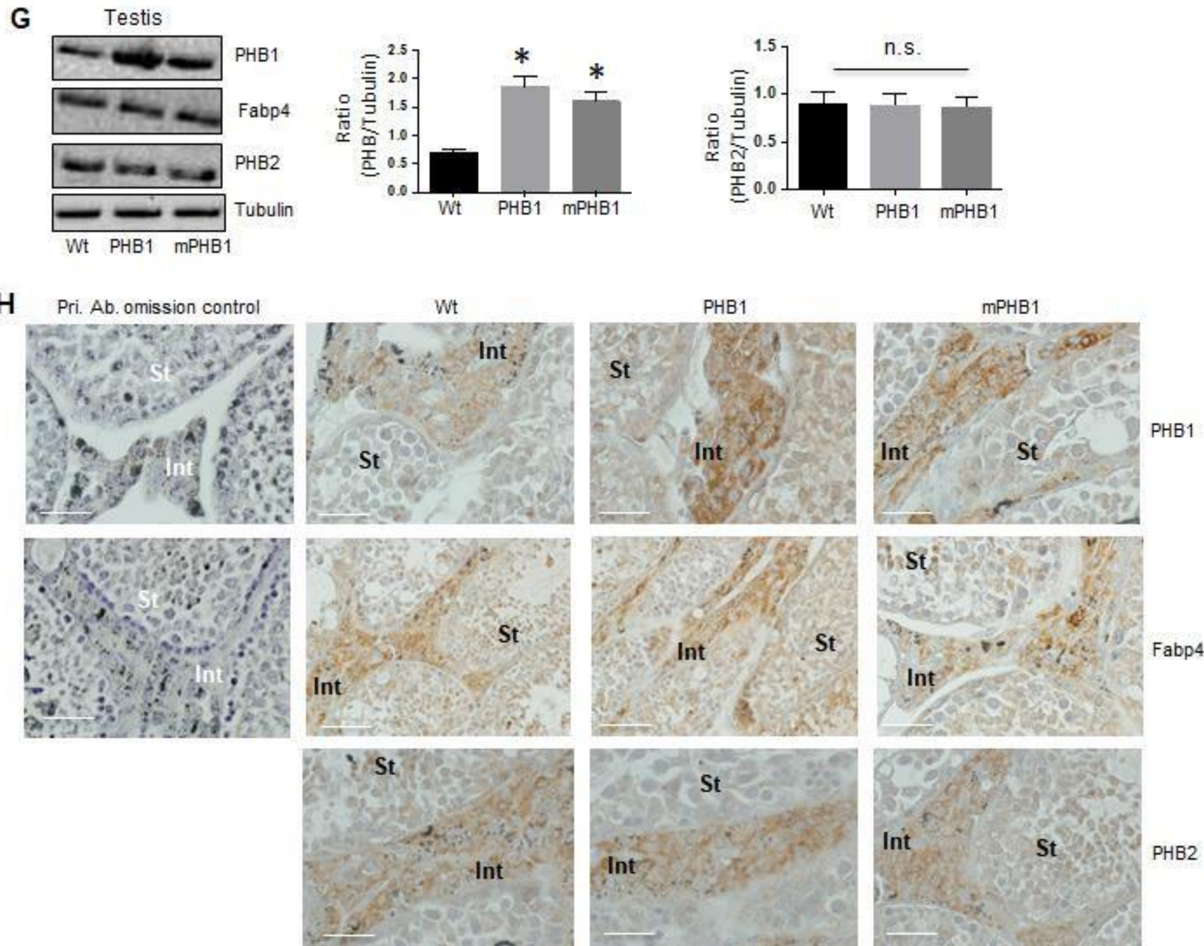
Next, we sought to uncover serum gonadotropin levels in mPHB1 and PHB1 mice, because of its role in the regulation of testis structure and functions, as well as its feedback relationship with testosterone [Miller et al., 2011]. Both LH and FSH, levels were significantly lower in mPHB1 mice compared with the PHB1 and wild-type mice (Fig. 4.1F) suggesting a potential negative feedback inhibition by higher testosterone levels in the mPHB mice. A significant difference in gonadotropin levels were also observed between the PHB1 and wild-type mice, which was lower in PHB1 mice (Fig. 4.1F). Collectively, a more pronounced hypogonadotropic hypogonadism phenotype of the mPHB1 mice when compared to the PHB1 mice would suggest that PHB1 plays a regulatory role in testosterone production by Leydig cells, which involves the Tyr^{114} residue in PHB1.



(F) Histograms depicting serum gonadotropin (LH, FSH) and progesterone levels in Wt, PHB1, and mPHB1 mice at 6 months of age.
*$p < 0.05$, **$p < 0.01$ between Wt and PHB1 or mPHB1 mice, # $p < 0.05$ between PHB1 and mPHB1 mice. Data are presented as mean ± SEM (n = 6 mice/group or experiments repeated atleast 3 times). n.s. – not significant, Wt – wild type.

4.3.2 PHB1 and mPHB1 mice overexpress PHB1 in their Leydig cells

The *Fabp-4* gene promoter that we used to develop the PHB1 and mPHB1 transgenic mice models is often used for adipocyte-specific gene manipulation because it is primarily expressed in adipocytes [Kusminski et al., 2012]. However, an unexpected testicular phenotype of the mPHB1 mice and increased testosterone production from the primary Leydig cells isolated from them raised a question about the potential expression of *Fabp-4* in the testis/Leydig cells, and consequently an overexpression of PHB1 or mPHB1 within them, contributing to a testicular phenotype, as observed in the PHB1 and mPHB1 mice (Fig. 4.1A-D). Subsequent research revealed that Fabp-4 was recently reported to express in the testis in mice, specifically in Leydig cells [O'Hara et al., 2015]. To find out if this is the case for PHB1 transgenic mice, I first analyzed testis samples from them using immunoblotting and immunohistochemistry. Fabp-4 protein was detected in the testis by both methods (Fig. 4.1G, H) and Leydig cell-specific expression of Fabp-4 was apparent from immunohistochemical analysis (Fig. 4.1H). Importantly, an increased expression of PHB1 was found in the testis samples using immunohistochemistry, especially in Leydig cells in the testicular interstitium of both transgenic mouse models compared with wild-type mice, matching the expression pattern of Fabp-4 (Fig. 4.1H). In addition, I examined the expression levels of PHB1's homologous protein, PHB2, which is known to form heterodimers with PHB1 in mitochondria. No difference in PHB2 protein levels were found in the testis samples from the PHB1-Tg and mPHB1-Tg mice compared with the wild-type mice (Fig. 4.1G, H). Together, these evidences confirmed that the PHB1 and mPHB1 mice overexpress PHB1 in their Leydig cells.



(G) Immunoblots showing PHB1, PHB2, and Fabp4 expression levels in testis from mice at 4 months of age. Quantification of PHB1 and PHB2 band densities are shown with histograms. Tubulin blot is included as a loading control.

(H) Photomicrographs depicting immunohistochemical analysis of PHBs and Fabp-4 in the testis from PHB1, mPHB1 and Wt mice at 4 months of age. The omission of primary antibodies is included as negative controls. Tissue sections were counterstained with hematoxylin for better visualization. Scale bar = 10 µm.

*p < 0.05, **p < 0.01 between Wt and PHB1 or mPHB1 mice, # p < 0.05 between PHB1 and mPHB1 mice. Data are presented as mean ± SEM (n = 6 mice/group or experiments repeated atleast 3 times, as applicable in panel A-G). n.s. – not significant, Pri. Ab. – primary antibody, Wt – wild type.

4.3.3 PHB1 is a LH-regulated protein and plays a role in hormone production by Leydig cells

To further explore PHB1's role in Leydig cell steroidogenesis, I resorted to MA-10 cells, a model murine Leydig cell line, which produces progesterone (P4) as a major product instead of testosterone [Ascoli, 1981]. A CRISPR/Cas9-mediated *Phb1* knockdown performed in MA-10 (CRISPR/Cas9-Phb-MA-10) cells significantly (Supplemenatry Fig. 1) decreased hCG-induced P4 production in comparison with control MA-10 cells (Fig. 4.2A), whereas overexpression of mPHB1 in CRISPR/Cas9-Phb-MA-10 cells not only rescued P4 production, but further enhanced in comparison with control group (Fig. 4.2A). A similar effect of shRNA-mediated Phb1 knockdown (Supplemenatry Fig. 1) on steroidogenesis was also observed in MA-10 cells in response to hCG stimulation (Fig. 4.2A). A similar outcome using two different experimental approaches would mean that the observed effect was specific to the manipulation of Phb1 levels in MA-10 cells. This finding prompted me to investigate whether PHB1 is a target gene for LH in Leydig cells, as LH plays a central role in almost every aspect of LC steroidogenesis [Medar et al., 2021]. The stimulation of LCs with hCG led to the upregulation of PHB1 protein in a time- and dose-dependent manner and displayed both acute (within 2hours) and chronic (36-48hours) effects (Fig. 4.2B). To further confirm acute regulation of PHB1 protein in Leydig cells, I repeated the experiment with dibutyryl cyclic-AMP (db-cAMP or cAMP) which is a known activator of PKA pathway, stimulation with db-cAMP in Leydig cells results in the synthesis and phosphorylation of StAR and production of testosterone. A similar effect on PHB1 levels was observed in response to db-cAMP (Fig. 4.2B). Thus, in my subsequent experiments, I used only db-cAMP (when reasonable) for consistency and to avoid batch variation in hCG preparations. An acute upregulation of PHB1 protein in response to hCG and db-cAMP during a 1 to 2-hour period (Fig. 4.2B) but not in mitochondrial protein Cox IV level would imply that augmented level of PHB1 is not due to an augmented number of mitochondria. This finding indicates a similarity with the regulation of StAR levels, which is known to acutely regulated in steroidogenic cells [Bose et al., 2002], including Leydig cells [Duarte et al., 2014 and Castillo et al., 2015]. Thus, I examined StAR levels using immunoblotting. Interestingly, a plausible relationship was observed between PHB1 and StAR levels in response to hCG stimulation, which was inversely related at first hour, 4 hours and 48 hours of the treatment and then both proteins showed similar pattern during other time points (Fig. 4.2B). Moreover, in response to db-cAMP, a dynamic change in StAR doublet bands were observed during the first four hours of stimulation showing a correlation with PHB1 protein

levels to some extent (Fig. 4.2B). In addition, I performed immunocytochemical analysis of PHB1 level in MA-10 cells in response to hCG and db-cAMP stimulation. Again, upregulation of PHB1 levels in response to steroidogenic stimulation was apparent in MA-10 cells (Fig. 4.2C). Our finding of acute regulation of PHB during first few hours of hCG stimulation led us to investigate whether PHB1 is regulated at the translational level in Leydig cells. For this, I stimulated MA-10 cells with hCG in the presence of cycloheximide (CHX), a protein synthesis inhibitor. A reduction in the expression level of PHB1 was observed (Fig. 4.2D), confirming that PHB1 is regulated at the translational level in Leydig cells. Collectively, this data confirmed that PHB1 is a gonadotropin-regulated protein in Leydig cells and plays a role in steroid hormone production by Leydig cells.

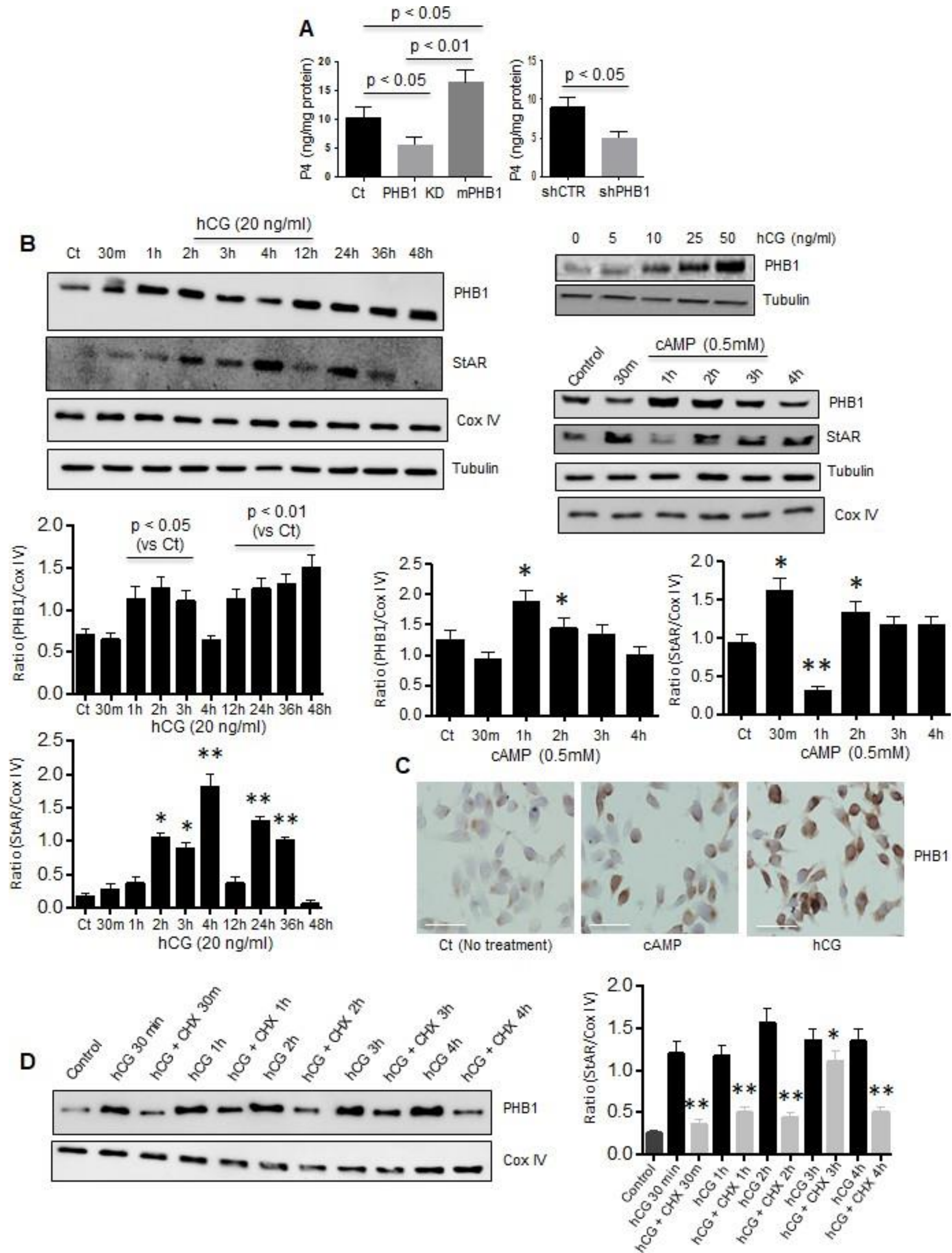

A
p < 0.05
p < 0.01
p < 0.05
P4 (ng/mg protein)
Ct
PHB1 KD
mPHB1
shCTR
shPHB1
B
hCG (20 ng/ml)
Ct 30m 1h 2h 3h 4h 12h 24h 36h 48h
PHB1
StAR
Cox IV
Tubulin
0 5 10 25 50 hCG (ng/ml)
cAMP (0.5mM)
Control 30m 1h 2h 3h 4h
Ratio (PHB1/Cox IV)
Ratio (StAR/Cox IV)
p < 0.05 (vs Ct)
p < 0.01 (vs Ct)
C
Ct (No treatment)
cAMP
hCG
D
Control
hCG 30 min
hCG + CHX 30m
hCG 1h
hCG + CHX 1h
hCG 2h
hCG + CHX 2h
hCG 3h
hCG + CHX 3h
hCG 4h
hCG + CHX 4h

Figure 4.2. PHB1 is a LH-regulated protein and plays a role in hormone production by Leydig cells

(A) Histograms showing the effect of CRISPR/Cas9- (left panel) and shRNA-mediated (right panel) PHB1 knockdown on hCG-induced progesterone production from MA-10 cells.

(B) Immunoblots showing time- and dose-dependent effects of hCG stimulation on PHB1 levels in MA-10 cells (left and upper right panel). Dose-dependent effect of db-cAMP on PHB1 levels in MA-10 cells (middle right panel). Cox-IV blot is shown as a loading control. Quantification of band intensities are shown with histograms (lower panel). Data are presented as mean ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ between Ct and cAMP or hCG stimulation (as applicable).

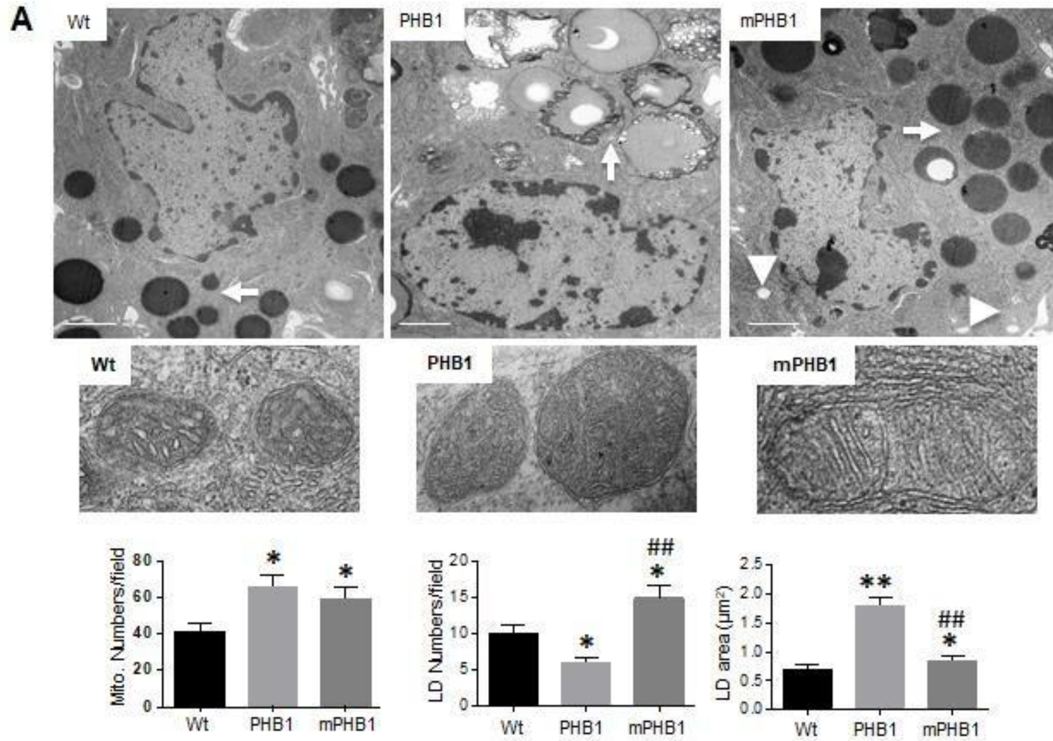
(C) Photomicrographs showing immunocytochemical analysis of PHB1 levels in MA-10 cells in response to 2 h treatment with db-cAMP and hCG. Scale bar = 10 μm.

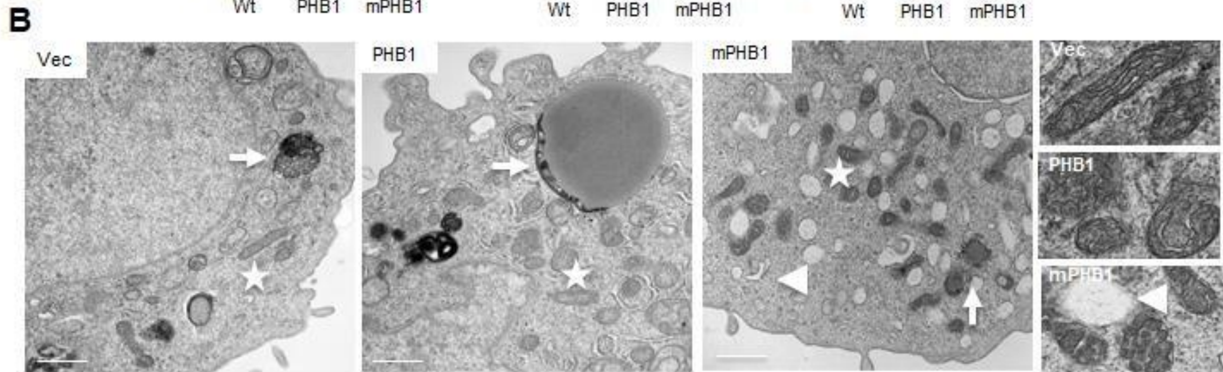
(D) Representative immunoblots showing the effect of cycloheximide (CHX) treatment on hCG induced changes in PHB1 levels in MA-10 cells. Cox-IV blot is shown as a loading control.
*$p <0.05$, **$p < 0.01$ between hCG and hCG + CHX.

4.3.4 Leydig cells from PHB1 and mPHB1 mice showed distinct mitochondrial and lipid droplet characteristics

There is much evidence in previous literature suggesting that PHB1 plays a wide-ranging and interconnected role in mitochondrial biology and lipid homeostasis [Osman et al., 2009; Merkwirth et al., 2008 and Osman et al., 2009]. For example, we have shown that transgenic mice overexpressing PHB1 in adipocytes develop obesity, which involves mitochondrial biogenesis [Osman et al., 2009], and others have reported that PHB1 has a role to play in mitochondrial phospholipid homeostasis in different model organisms [Osman et al., 2009; Merkwirth et al., 2008 and Osman et al., 2009]. Furthermore, the PHB1 family member MEC-2 binds cholesterol in relation to membrane signaling and functions [Huber et al., 2006], whereas Erlin-1 and Erlin-2 are highly enriched in the detergent-soluble ER fraction in a cholesterol-dependent manner [Browman et al., 2006]. Thus, a possibility exists that PHB1 and mPHB1 may influence mitochondrial biology and cholesterol homeostasis within Leydig cells, and this could be the reason for the increased serum testosterone levels observed in the PHB1 transgenic mice (Fig. 4.1B) and in primary Leydig cells derived from them (Fig. 4.1D). Therefore, I analyzed testis samples from the PHB1 transgenic mice using transmission electron microscopy (TEM). A substantial increase in lipid droplets (LDs) was observed in Leydig cells from both the PHB1 and mPHB1 mice compared with the wild-type mice (Fig. 4.3A). However, a difference in size, number, and morphology of the lipid droplets (LDs) were apparent in Leydig cells between them (Fig. 4.3A). In Leydig cells of the PHB1 mice, the lipid droplets were significantly larger, irregular in shape, and showed signs of lysosomal degradation, whereas Leydig cells from the mPHB1 mice displayed significantly smaller, uniform, and regular lipid droplets, indicating a difference in lipid processing (Fig. 4.3A). In addition, a difference in mitochondrial shape was observed between the PHB1 and mPHB1 mice, which were primarily oval or circular in Leydig cells from the PHB1 mice, whereas they appeared fusiform or elongated in Leydig cells from mPHB1 mice (Fig. 4.3A). Moreover, a difference in mitochondrial cristae were noticeable, which were more prominent in Leydig cells from the mPHB1 mice compared with the PHB1 mice (Fig. 4.3A). In addition to structural differences, an increase in mitochondrial density was found in Leydig cells from the PHB1 and mPHB1 mice compared with the wild-type mice (Fig. 4.3A). Moreover, a similar change in lipid droplets and mitochondrial features were observed in PHB1 and mPHB1-overexpressing MA-10 cells (Fig. 4.3B). In addition, a substantial increase in lysosome population

was observed in mPHB1 expressing MA-10 cells (Fig. 4.3B). Importantly, performing a PHB1 knockdown in MA-10 cells led to the dysregulation of mitochondrial structure (e.g., fragmentation of mitochondrial cristae) and lipid droplets (Fig. 4.3C). Collectively, this data indicates that PHB1 and mPHB1 have different effects on lipid/cholesterol handling, mitochondrial structure and functions in Leydig cells, which may be direct effects at the mitochondrial level, or perhaps indirect effects due to changes in upstream signaling events and impaired lipid handling.


A
Wt
PHB1
mPHB1
Wt
PHB1
mPHB1
Mito. Numbers/field
LD Numbers/field
LD area (μm²)
Wt
PHB1
mPHB1


B
Vec
PHB1
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mPHB1

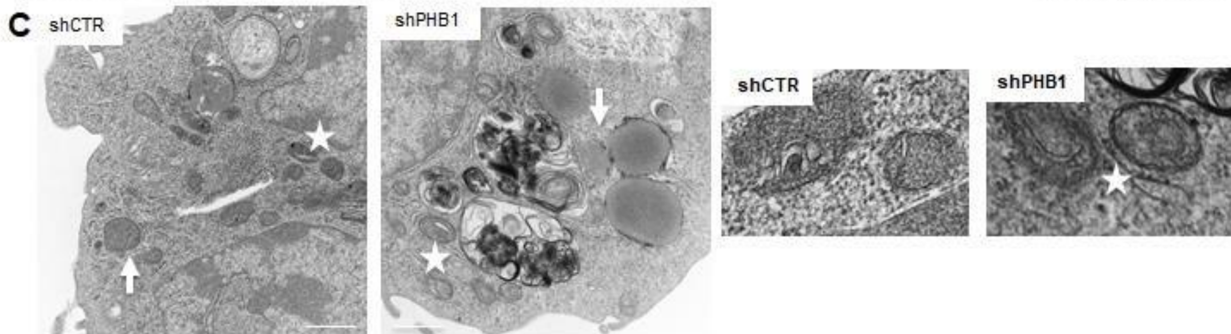

C
shCTR
shPHB1
shCTR
shPHB1

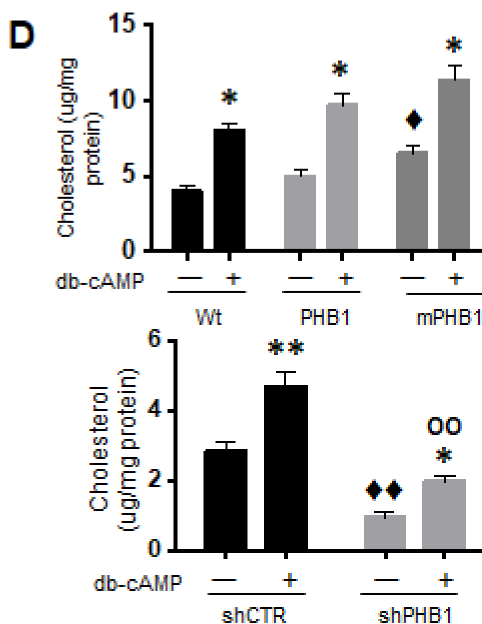
Figure 4.3. Leydig cells from PHB1- and mPHB1 mice showed distinct mitochondrial and lipid droplet characteristics

(A) Photomicrographs depicting TEM analysis of Leydig cells in the testis from PHB1, mPHB1 and Wt mice (upper panel, Scale bar = 500 nm) and magnified (46000x) view of their mitochondria (middle panel). Histograms depicting mitochondrial number, as well as and lipid droplet size and number in Leydig cells (lower panel).

*p < 0.05, **p < 0.01 between Wt and PHB1 or mPHB1 mice, # p< 0.05, ## p < 0.01 between PHB1 and mPHB1 mice. Data are presented as mean ± SEM (n=6).

(B & C) Photomicrographs depicting TEM analysis of PHB1 manipulated MA-10 cells (Scale bar = 500 nm) and magnified view of their mitochondria (right panel in B and lower panel in C) are also shown for better visualization. Star indicates mitochondria, arrowhead indicates lysosomes, and arrow indicated lipid droplets. Data are presented as mean ± SEM (n=6).

Next, I measured cholesterol levels in primary Leydig cells isolated from the testis samples of the PHB1 and mPHB1 mice. Cholesterol levels were found to be significantly higher in Leydig cells from both the PHB1 and mPHB1 mice when compared with the wild type mice under a basal state ($p<0.01$); however, the difference between the PHB1 mice and mPHB1 mice was not significant (Fig. 4.3D). In response to cAMP stimulation, a significant increase in cholesterol levels was observed in all groups in comparison with the unstimulated respective control groups (Fig. 4.3D). However, the amplitude of increase was maximum in Leydig cells from the mPHB1 mice and minimum in Leydig cells from the wild-type mice (Fig. 4.3D) suggesting that Leydig cells from the mPHB1 mice are more efficient in cholesterol uptake in response to steroidogenic stimulation. Importantly, shRNA-mediated knockdown of PHB1 in MA-10 cells led to significant decrease in cholesterol uptake under basal and stimulated condition in comparison with scramble shRNA transfected control cells (Fig. 4.3D). Together, this data suggests that PHB plays a role in cholesterol uptake and handling in Leydig cells.



(D) Histograms showing cholesterol levels in Leydig cells from PHB1, mPHB1 and Wt mice (upper panel) and in PHB1 manipulated MA-10 cells (lower panel) in response to db-cAMP stimulation. *$p < 0.05$, **$p < 0.01$ between stimulated and unstimulated cells, ♦$p<0.05$ Wt vs. mPHB1 (basal), ♦♦$p<0.01$ shCon vs. shPHB1 (basal) and $^{oo}p<0.01$ shCon vs. shPHB1 (stimulated). Data are presented as mean ± SEM (n=3 mice/group or experiment repeated 3 time, as applicable). shCTR – scramble control shRNA, shPHB1 – PHB1 shRNA.

4.3.5 PHB1 contains putative cholesterol binding motifs and interacts with cholesterol

A potential link between PHB1 and lipid / cholesterol homeostasis in Leydig cells (as revealed by TEM analysis and cholesterol uptake in PHB1-manipulated MA-10 cells) prompted us to examine PHB1's protein sequence for putative cholesterol binding domains or motifs [Romanowski et al., 2002 and Yang et al., 2014]. An analysis of PHB1's protein sequence using the NCBI BLAST tool displayed that PHB1 lacks the cholesterol-binding domain present in the StAR family of proteins; however, a number of putative Cholesterol Recognition Amino Acid Consensus sequences (generally referred to as the CRAC motif) and inverted CRAC sequences (generally referred to as the CARC motif) were identified (Fig. 4.4A). Both PHB1 and its heterodimeric partner PHB2 were found to contain multiple cholesterol binding motifs, including motifs spanning the conserved tyrosine residues (Fig. 4.4A). To determine whether putative cholesterol-binding motifs in PHB1 interacts with cholesterol, I performed a cholesterol-binding assay using cholesterol-immobilized beads and MA-10 cell lysates (with/without hCG stimulation). Both, PHB1 and PHB2 were successfully pulled down by the cholesterol beads but evaded the control beads (Fig. 4.4B). This data suggests that PHB1 and PHB2 contain multiple cholesterol-binding motifs, which appears to interact with and bind cholesterol.

A CARC Motifs (K/R)-X1-5-(Y/F)-X1-5-(L/V) in PHB1 (NP_032857.1) & PHB2 (NP_031557.2)

PHB1		PHB2	
35	RAVIFDRFRGV	38	RESVFTV
93	RILFRPV	48	RAIFFNRIGGV
128	KSVVARFDAGEL	142	KSVVAKFNASQL
156	RAAT**FGLI**	171	RAKD**FSLIL**
177	KEFTEAV	191	REYTAAV
195	RARFVV	209	RAQFLV
255	RNITYL	270	RIYLTADNL

CRAC Motifs (L/V)-X_{1-5}-(Y)-X_{1-5}-(K/R) in PHB2

32 VAYGVR
117 LPSM**YQRL**GLDYEER
242 LSKNPGYIKLRK

CRAC/CARC motifs overlapping LC3 motifs

PHB1 103 LPRI**YTSI**GEDYDER
PHB2 117 LPSM**YQRL**GLDYEER
PHB1 156 RAAT**FGLIL**
PHB2 171 RAKD**FSLIL**

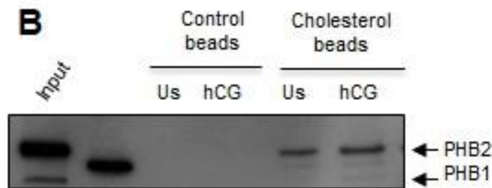


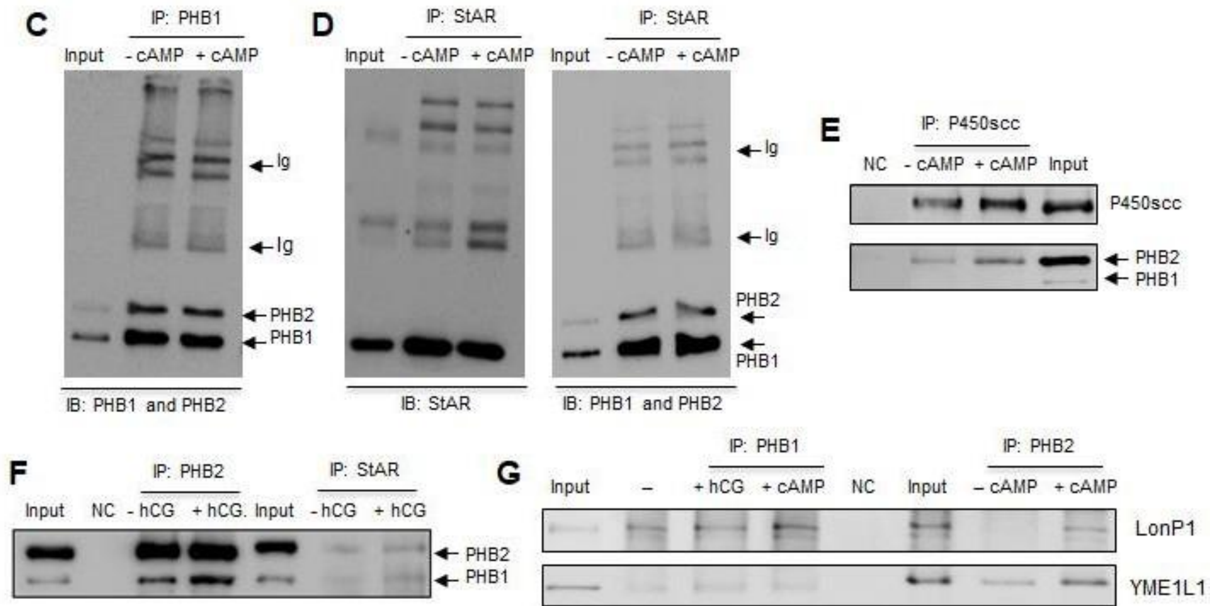
Figure 4.4. PHB1 contains putative cholesterol binding motifs, interacts with StAR and P450scc, and forms heterodimeric megacomplex with PHB2 in Leydig cells

(A) Multiple PHB1 and PHB2 peptide sequences containing putative CRAC and CARC motifs. Overlapping LC3 binding motif in PHB1 and PHB2 are shown in bold.

(B) Immunoblots depicting PHB1 pulldown using cholesterol immobilized agarose beads. Only agarose beads (without cholesterol) were included as negative control and recombinant PHB128-272 (second lane) as a positive control. Us: unstimulated,

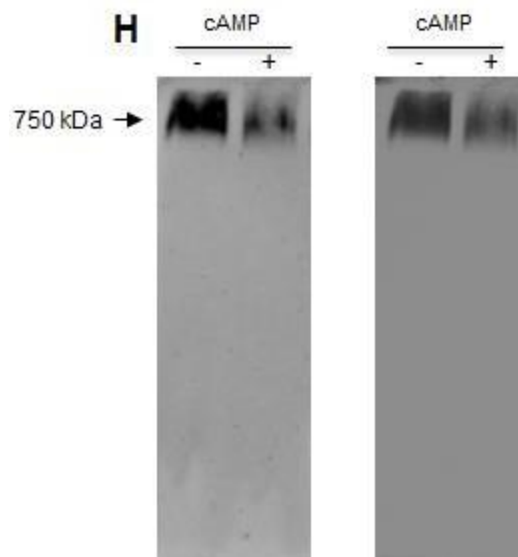
4.3.6 PHB1 interacts with StAR and P450scc and forms a heterodimeric megacomplex with PHB2 in Leydig cells

PHB1 and its homologous protein PHB2 form a heterodimeric megacomplex spanning the inner mitochondrial membrane [Tatsuta et al., 2005] (where P450scc resides) and have been identified as phosphoproteins along with StAR in rat granulosa cells (a steroidogenic cell type in the ovary) [Thompson et al., 1997]. Thus, PHB1 heterodimeric megacomplex may potentially interact with proteins involved in cholesterol transport and utilization across mitochondrial membranes (e.g., StAR and P450scc). To explore this possibility, I immunoprecipitated PHB1 using PHB1 antibody that detects endogenous levels of total PHB1 protein from cAMP-stimulated and unstimulated MA-10 cell lysates using protein-specific monoclonal antibodies and Dynabeads. As expected, PHB2 was co-immunoprecipitated with PHB1 (Fig. 4.4C). Next, we immunoprecipitated StAR and P450scc using a protein-specific antibody. Both, PHB1 and PHB2 were co-immunoprecipitated with StAR (Fig. 4.4D), whereas only PHB2 was found to be co-immunoprecipitated with P450scc (Fig. 4.4E). Interestingly, PHB2 was co-immunoprecipitated with both P450scc and StAR (Fig. 4.4D, E). Thus, I immunoprecipitated PHB2 using PHB2 monoclonal antibody that detects endogenous levels of total PHB2 protein similarly and analyzed the results using immunoblotting. Only PHB1 was co-immunoprecipitated with PHB2 (Fig. 4.4F). In mitochondria, lonP1 (serine peptidase) and YME1L-1 (ATP dependent metalloprotease) are essential to maintain mitochondrial quality by balancing mitochondrial fission and fusion. As our findings are suggestive of an inverse and dynamic relationship between StAR and PHB1 protein levels during their acute regulation (Fig. 4.2B), we examined the potential interaction of mitochondrial proteases with PHB1 and PHB2 because they are known to interact or regulate them [Osman et al., 2009 and Merkwirth et al., 2008]. Both LonP1 and YME1L-1 were co-immunoprecipitated with PHBs, and their band intensities were relatively higher under db-cAMP-stimulated conditions (Fig. 4.4G) suggesting the potential interactions between mitochondrial proteases and PHBs in the acute regulation of StAR.



(C) Immunoblots depicting co-immunoprecipitation of PHB1 heterodimeric partner PHB2 from MA-10 cells (-/+ db-cAMP treatment, 0.5mM) as determined by respective protein-specific antibody. Only a small amount (~1/5th) was used in input lane to avoid band saturation and potential spill over to the adjacent lane.
(D) Immunoblots showing co-immunoprecipitation of PHBs with StAR from MA-10 cells (-/+ db-cAMP stimulation, 0.5mM). Again, only a small amount (~1/5th) was used in input lane.
(E) Immunoblots showing co-immunoprecipitation of PHBs with P450scc from MA-10 cells with or without db-cAMP treatment (0.5mM).
(F) Immunoblots showing co-immunoprecipitation of PHB1 with PHB2 from MA-10 cells with or without hCG stimulation (20ng/ml).
(G) Immunoblots showing co-immunoprecipitation of mitochondrial proteases with PHB1 and PHB2 from MA-10 cells with or without hCG (20ng/ml) or db-cAMP treatment (0.5mM).

To confirm whether PHB1 and PHB2 form a heterodimeric megacomplex in Leydig cells, I analyzed cell lysates prepared from db-cAMP-stimulated and unstimulated cells using BN-PAGE and immunoblotting. The heterodimeric complex of PHBs was detected by both protein-specific antibodies, confirming their formation in MA-10 cells (Fig. 4.4H). Notably, the band density of the megacomplex was found to be relatively less intense under stimulated conditions (Fig. 4.4H) indicating a potential importance of the dynamics of the heterodimeric complex and their interaction with other partners in Leydig cells in response to steroidogenic stimulation. Collectively, this data suggests that the PHB1 and PHB2 heterodimers interact with StAR, mitochondrial proteases, and P450scc in MA-10 cells.



(H) Immunoblots showing heterodimeric megacomplex of PHB1 and PHB2 in MA-10 cells with or without db-cAMP treatment, as determined by BN-PAGE and immunoblotting.
All experiments were repeated for at least 3 times. IB – immunoblotting, IP – immunoprecipitation, Ig – Immunoglobulin band, NC – negative control

4.3.7 PHB1 modulates PKA and ERK signaling in Leydig cells

Previously, we discovered the phosphorylation of PHB1 at the Tyr^{114} residue occurs in relation to insulin signaling [Ande et al., 2009; Ande and Mishra, 2009], and have shown its importance in adipocyte differentiation, including a modulatory role in MAPK-ERK signaling in a context-dependent manner [Ande et al., 2012]. Moreover, works by others have shown that PHB1 undergoes phosphorylation at the Tyr^{114} residue in many cell types in relation to growth factors (IGF, EGF) [Rajalingam et al., 2005], hormones (FSH) [Chowdhury et al., 2013] and diverse immune signaling pathways [Kim et al., 2013; Ande et al., 2016]. Thus, I examined the activation level of cell-signaling pathways (i.e., cAMP-PKA and MAPK-ERK) in the testis samples taken from the PHB1-Tg and mPHB1-Tg mice. As the cAMP-PKA signaling pathway plays a central role in mediating the LH response in Leydig cells [Medar et al., 2021], I first investigated the phospho-PKA (p-PKA) in testicular lysates by immunoblotting using a phospho-specific antibody. A relatively higher p-PKA level was found in the testis samples from the mPHB1-Tg mice compared to the PHB1-Tg and wild-type mice (Fig. 4.5A). In addition to PKA, the MAPK-ERK pathway plays a role in LH signaling in Leydig cells [Duarte et al., 2014]. As PHB1 modulates MAPK-ERK signaling, we determined the phospho-ERK1/2 (p-ERK1/2) levels in the testis samples from the PHB1-Tg and mPHB1-Tg mice by immunoblotting using a phospho-specific antibody. The p-ERK1/2 level was found to be significantly upregulated in the mPHB1-expressing testis samples compared to the PHB1-expressing and control testis samples from the wild-type mice (Fig. 4.5A). However, such a difference in p-ERK1/2 levels was not found between the testis from the PHB-overexpressing and wild-type mice (Fig. 4.5A). This data indicates that PHB1 plays a regulatory role in the regulation of basal cAMP-PKA and MAPK-ERK signaling in the testis/Leydig cells, which require the Tyr^{114} residue in PHB1 as its substitution for mPHB1 leads to increased p-ERK1/2 levels. Moreover, the effect mPHB1 on p-ERK1/2 was relatively more apparent than the p-PKA levels (Fig. 4.5A). To further validate PHB1's role in the modulation of ERK phosphorylation, I transfected CRISPR/Cas9-Phb-MA-10 cells with different PHB1 constructs and examined the effect of hCG stimulation on pERK1/2 levels. Again, an increased pERK1/2 level was observed in MA-10 cells expressing mPHB1 in comparison with PHB1 and only vector-transfected cells (Fig. 4.5B) confirming PHB1's role in the regulation of pERK levels. Although a similar trend in p-PKA level was observed between PHB1 and mPHB1-expressing cells; however, the effect was not apparent like p-ERK1/2 levels (Fig. 4.5B).

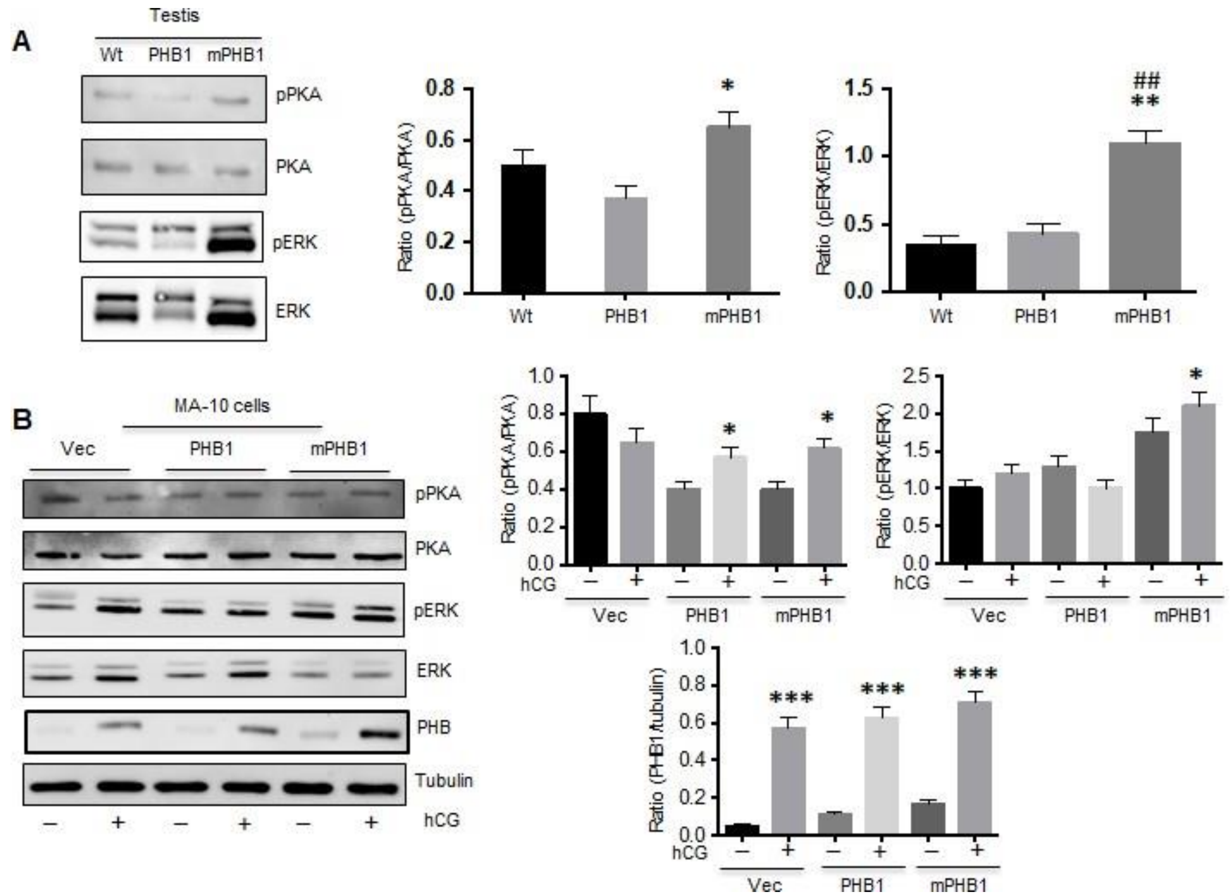


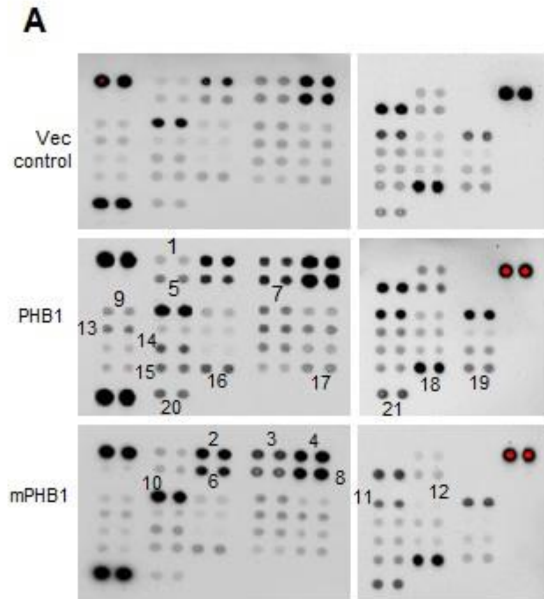
Figure 4.5. PHB1 modulates PKA and ERK signaling in Leydig cells

(A) Immunoblots showing pPKA and pERK1/2 levels in the testis from PHB1, mPHB1 and Wt mice (left panel). Quantification of protein band densities are shown by histograms (middle and right panels). *p < 0.05, **p < 0.01 between Wt and PHB1 or mPHB1 mice, ## p < 0.01 between PHB1 and mPHB1 mice. Data are presented as mean ± SEM (n=3).

(B) Immunoblots showing pPKA and pERK1/2 levels in MA-10 cells (with or without hCG stimulation) transfected with different PHB1 constructs (left panel). Vector only transfected cells were used as a control. Tubulin blot is shown as a loading control. Histograms showing quantification of protein band densities (right panel).

*p < 0.05, ***p < 0.001 between unstimulated and stimulated cells in each experimental group. Data are presented as mean ± SEM (n = 3).

Next, I used a Phospho Kinase Array (containing 43 kinase phosphorylation sites and 2 related proteins – cAMP response element binding protein (CREB) and p53) to get a snapshot of various kinases and cell signaling molecules in response to db-cAMP in PHB1 and mPHB1 expressing MA-10 cells. A total of 19 signaling molecules were found to be upregulated and 2 (i.e., JNK and WNK1 kinases) were downregulated in PHB1/mPHB1 overexpressing cells when compared with control group (Fig. 4.6A, B). Interestingly, 10 of them were differentially altered in PHB1 and mPHB1 expressing cells and most of them were substantially downregulated in mPHB1 when compared with PHB1 expressing cells (Fig. 4.6A, B). As expected, out of 21 altered kinase phosphorylation, 16 are known to play a role in Leydig cells, 14 in steroidogenesis, and all of them are known to be involved in the mitochondrial biology (Fig. 4.6C) suggesting that PHB1 plays an important role in cell signaling and mitochondrial biology in Leydig cell pertaining to steroidogenesis. Notably, cAMP regulated transcription factor CREB and mediators of MAPK pathway (e.g., ERK and JNK), which plays a critical role in gonadotropin-induced chronic steroidogenesis was found to be substantially upregulated in PHB1 and mPHB1 overexpressing MA-10 cells (Fig. 4.6A, B).



C

Kinase or related proteins	LC functions / Steroido.	Mitochondrial functions
1. p38α	No / Yes	Yes
2. ERK1/2*	Yes / Yes	Yes
3. JNK1/2/3	Yes / Yes	Yes
4. GSK3α/β	Yes / Yes	Yes
5. EGFR	Yes / Yes	Yes
6. MSK	No / No	Yes
7. AMPKα1	Yes / Yes	Yes
8. Akt1/2	Yes / Yes	Yes
9. TOR	Yes / Yes	Yes
10. CREB*	Yes / Yes	Yes
11. P70 S6	Yes / Yes	Yes
12. p53	Yes / Yes	Yes
13. Src	Yes / Yes	Yes
14. Yes	Yes / Yes	Yes
15. Chk2	Yes / No	Yes
16. FAK	Yes / No	Yes
17. STAT5b	Yes / No	Yes
18. WNK1	No / No	Yes
19. PYK2	No / No	Yes
20. PRAS40	No / No	Yes
21. HSP60	Yes / Yes	Yes

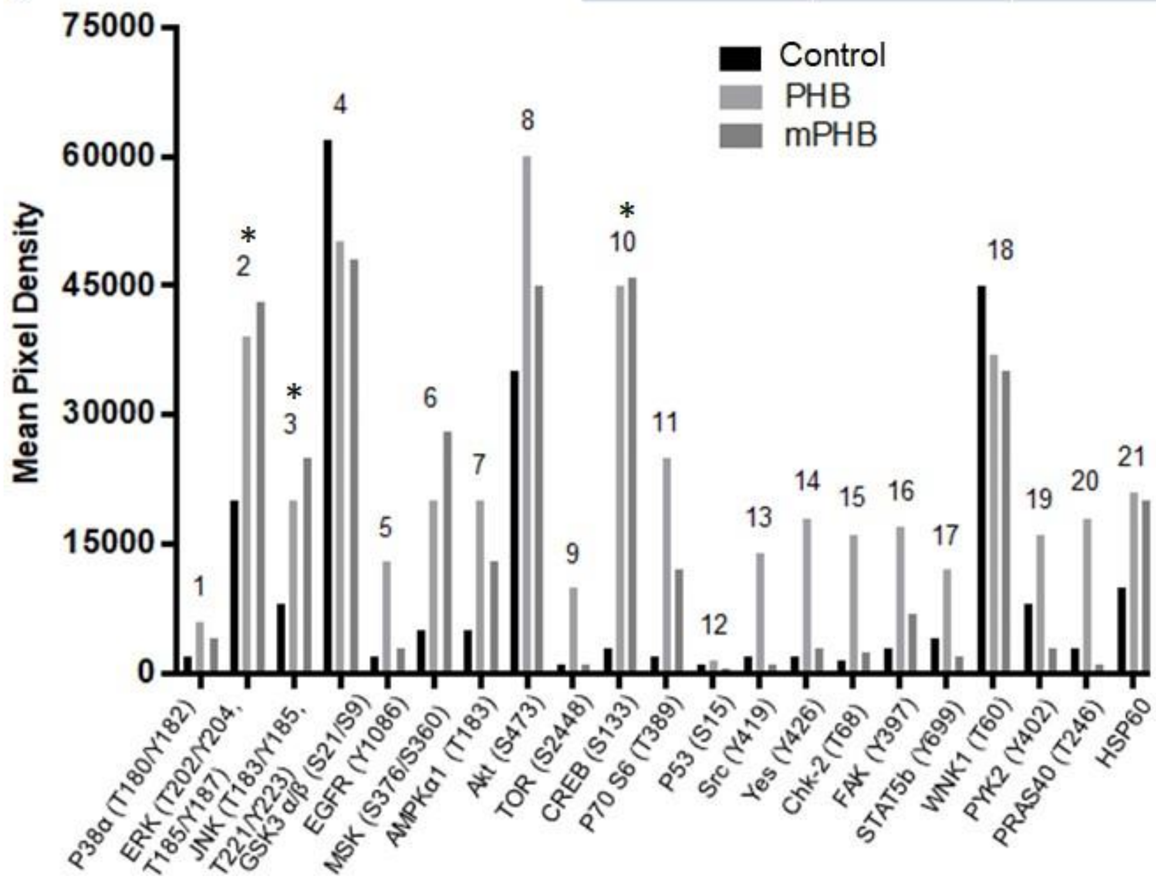


Figure 4.6. Phosphokinase array profiling of PHB1 and mPHB1 transfected MA-10 cells in response to hCG

(A) Phosphokinase array blots showing differential phosphorylation levels of various signaling molecules in MA-10 cells overexpressing PHB1 and mPHB1 in response to hCG stimulation (20ng/ml for 30m). Vector only transfected cells were used as a control.

(B) Histograms showing comparison of signals on different arrays depicting relative change in phosphorylated kinase proteins between different experimental groups.

(C) List of kinase or related proteins identified by Phosphokinase arrays and their status in relation to Leydig cell (LC) function, steroidogenesis and mitochondrial functions based on current literature (PubMed). Steroido – steroidogenesis.

* Indicates mediators of PKA and MAPK signaling pathways.

Thus, I examined the expression levels of steroidogenic marker proteins in the testis samples from the PHB1 and mPHB1 mice. As hypothesized, steroidogenic marker protein (3β-HSD and 17β-HSD) levels were significantly higher in transgenic mice sample compared with wild-type mice (Fig. 4.7A), which further support my findings of higher testosterone levels in PHB1 transgenic mice. In addition, a consistent upregulation of MAPK-ERK pathway in the testis samples and PHB1/mPHB1 manipulated MA-10 cells would imply its role in the effect of mPHB1 in Leydig cells and testicular phenotype in mPHB1-Tg mice. Thus, I investigated the effect of PHB1 knockdown on hCG-induced ERK1/2 phosphorylation in MA-10 cells. The pERK1/2 level was found to be significantly higher in PHB1 knockdown and mPHB1 expressing cells compared with control MA-10 cells (Fig. 4.7B) further supporting my conclusion that PHB1 plays a context-dependent regulatory role in Leydig cells under basal and steroidogenic induction. This finding prompted me to explore the role of pERK in mediating mPHB1 induced enhanced steroidogenesis in Leydig cells. Incubation of mPHB1 expressing CRISPR/Cas9-Phb-MA-10 cells with MAPK/ERK inhibitor (U0126) reversed mPHB1-induced P4 production in response to hCG (Fig. 4.7C) suggesting its role in mPHB1-induced upregulation of steroidogenesis in Leydig cells.

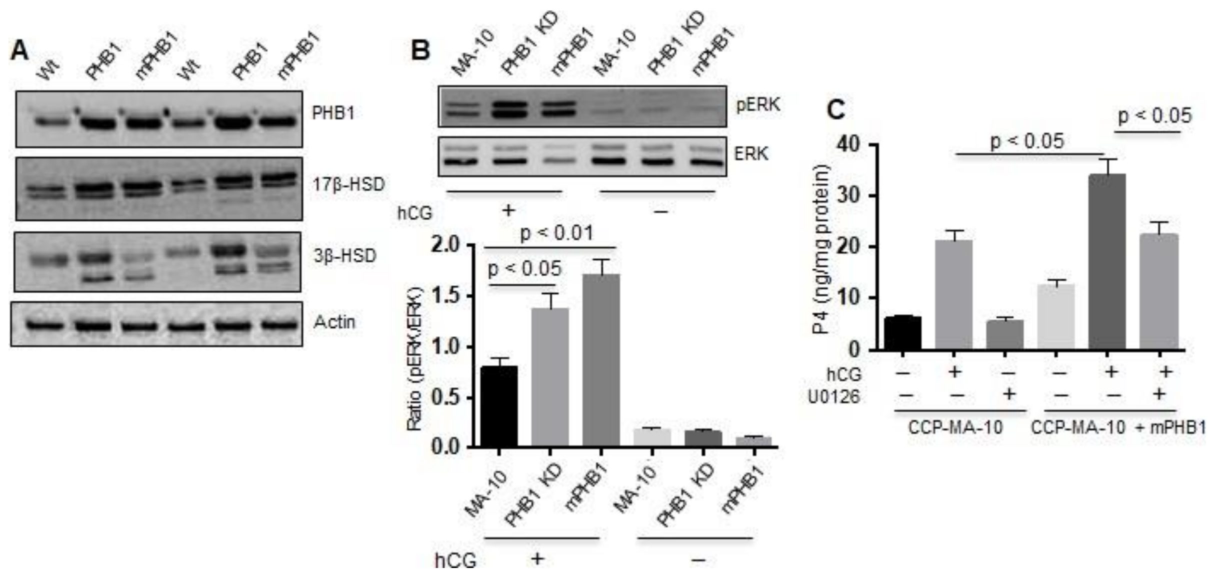


Figure 4.7. Steroidogenic markers are upregulated in the testis from PHB1 transgenic mice and PHB1 modulates ERK phosphorylation in a context-dependent manner
(A) Immunoblots showing the expression levels of steroidogenic marker proteins in the testis from wild-type and transgenic mice (n = 3).
(B) Upper panel: Immunoblots depicting pERK levels in PHB1 manipulated MA-10 cells in response to hCG. Lower panel: Histograms depicting quantification of band intensities (n = 3).
(C) Histograms showing the effect of MAPK/ERK inhibitor (U0126, 7nM)) on mPHB1-induced P4 production from CRISPR/Cas9-Phb-MA-10 (CCP-MA-10) cells in response to hCG (n = 3).

4.4 Discussion

This study reports that an evolutionarily conserved pleiotropic protein named PHB1 plays a multifaceted role in Leydig cell steroidogenesis, spanning the cytosolic and the mitochondrial compartments. This includes a role in hormone-induced cell signaling, intracellular cholesterol homeostasis, and the functional coupling of StAR and P450scc across the mitochondrial membrane. A higher testosterone level in the mPHB1 mice compared with the PHB1 and wild-type mice, and a similar finding from Leydig cells isolated from them, as well as in PHB1/ mPHB1-manipulated MA-10 cells implies that PHB1 plays a regulatory role in Leydig cell steroidogenesis, which involves the Tyr^{114} residue in PHB1, as its substitution in mPHB1 leads to increased steroidogenesis and consequently reduced gonadotropin levels. The mPHB1-related increased steroidogenesis in Leydig cells appears to involve augmented pERK signaling, intracellular cholesterol handling and mitochondrial attributes. In aggregate, my findings indicate that a coordination between cell signaling, and mitochondrial functions is necessary in controlling steroidogenesis in Leydig cells (i.e., to prevent steroid insufficiency or excess), which have effects on the negative feedback regulation of gonadotropin at the pituitary level.

Of note, PHB1 has been reported to play a role in ovarian granulosa cells (GCs). However, the focus of these studies was on granulosa cell proliferation, differentiation, survival, and atresia/apoptosis rather than steroidogenesis [Chowdhury et al., 2013; Chowdhury et al., 2007; Chowdhury et al., 2016; Wang et al., 2013; Wang et al., 2013 and Thompson et al., 2004], likely because of existing knowledge of PHB1's context-dependent role in cell proliferation, survival, and apoptosis in different cell types. For example, Chowdhury et al., 2013 have reported that the administration of equine chorionic gonadotropin (eCG) increases PHB1 expression in the ovarian follicles and GC, but not in theca-interstitial cells within the pre-antral follicles. This increased expression of PHB1 corresponded with follicular growth and decreased after the ovulatory luteinizing hormone (LH) surge and during follicular atresia. This finding would imply that the LH surge during the ovarian cycle may negatively regulate PHB1 expression. Moreover, a change in the phosphorylation levels of PHB1 and increased trafficking to the mitochondria was observed. Notably, the PHB1 phosphorylation sites under these culture conditions in response to FSH and testosterone were the Tyr^{249}, Thr^{258} and Tyr^{259} sites [Chowdhury et al., 2007 and Chowdhury et al., 2016], which we have reported in relation to insulin signaling and lipid binding/metabolism

[Ande et al., 2016; Ande et al., 2012; Ande et al., 2014; Ande et al., 2009; Ande and Mishra, 2009]. Intriguingly, Wang et al [2013] have reported an inhibitory effect on granulosa cell steroidogenesis whereas Choudhury et al [2007, 2013, and 2016] have reported an opposite, which may be stage-dependent role of PHB1 in granulosa cell biology and would require further investigations. Taken together, a possibility exists that the phosphorylation of PHB1 may play a role in steroidogenic cells in response to hormones and growth factors (e.g., trophic hormones, insulin, IGF and EGF), which are known to stimulate steroidogenesis. In summary, PHB1 possesses many features that may potentially contribute to steroidogenesis in different steroidogenic cell types.

Cholesterol serves as an essential substrate for all steroid hormones and its trafficking and homeostasis in steroidogenic cells are tightly regulated [Rone et al., 2009 and Elustondo et al., 2017]. Because of a highly hydrophobic chemical property, the cellular uptake and intracellular trafficking of cholesterol is mediated through different proteins [Rone et al., 2009]. Proteins that interact with cholesterol often contain cholesterol-binding domains or motifs [Romanowski et al., 2002 and Yang et al., 2014]. For example, the StAR family members contain the START (StAR-related lipid-transfer) domain, which binds hydrophobic lipids [Tsujishita et al., 2000], whereas P450scc contains the CRAC and CARC short linear motifs [Midzak et al., 2011]. The central tyrosine residue in the CRAC motif (L/V-X1-5-Y-X1-5-K/R) is crucial for cholesterol binding [Rone et al., 2009 and Elustondo et al., 2017]. The CARC motif (K/R-X1-5-Y/F-X1-5-L/V) is similar to the CRAC motif but exhibits the opposite orientation along the polypeptide chain from the N-terminus to the C-terminus [Rone et al., 2009 and Elustondo et al., 2017]. In addition to the reverse orientation, CARC is distinct from CRAC in that the central aromatic amino acid can be either Tyr or Phe [Rone et al., 2009]. Thus, it is possible that $PHB1^{Tyr114Phe}$ may retain the cholesterol-binding function of PHB1 and may contribute to differences in lipid droplet characteristics and steroid hormone production, as observed in Leydig cells from the PHB1 and mPHB1 mice. Moreover, previously our lab has reported that Tyr^{114} in PHB is a part of other lipid-binding motifs [Ande SR and Mishra S, 2009], and other PHB1 family members have been reported to bind cholesterol and be involved in lipid homeostasis [Osman et al., 2009 and Merkwirth et al., 2008], including mitochondrial phospholipid metabolism [Osman et al., 2009 and Merkwirth et al., 2008]. Thus, a difference in lipid droplet characteristics in Leydig cells from the PHB1 and m-PHB1 mice, as revealed by TEM and their relationship with steroid hormone production, would imply that the Tyr^{114} residue plays a role in it. As lipid droplets in Leydig cells

primarily contain cholesterol and cholesterol esters, this would mean that Leydig cells from the PHB1 and mPHB1 mice differ in cholesterol handling, which may include uptake, storage, transport, and their subsequent utilization for steroidogenesis. Of note, the ultrastructural features of Leydig cells from the PHB1 mice were very similar to previous reports from *StAR* [Ishii et al., 2002] and *Cyp11A1* knockout mice [Chein et al., 2013], such as increased lipid accumulation and signs of lipid droplet degradation. However, unlike *StAR* and *Cyp11A1* knockout mice, as well as according to a histopathology of naturally occurring mutations in the StAR gene in humans [Miller et al., 1997], the PHB1 and mPHB1 mice do not display high trophic hormone levels. Thus, the dysregulation of lipid/cholesterol homeostasis in Leydig cells from the PHB1 and mPHB1 mice is likely due to Leydig cell-specific alterations independent of their LH levels, which is substantially lower in comparison with the wild-type mice. It is possible that the overexpression of PHB1/ mPHB1 in Leydig cells of the PHB1/mPHB1 mice leads to a change in Leydig cell-specific attributes involved in cholesterol handling, including uptake, storage, mobilization, and utilization. However, a difference in testosterone production between the PHB1 and mPHB1 mice may be due to the upregulation of a mitochondria-specific function of PHB1 involving Tyr^{114}, which is independent of its role in pERK signaling. Thus, Tyr^{114}'s regulatory role may involve membrane signaling or mitochondrial functions, or a combination of both. Of note, the cholesterol binding motifs in PHB1 and the recently identified LC3 binding motifs [Wei et al., 2017] overlap with each other. This raises the important question of whether these two features of PHB1 work in a mutually exclusive and context-dependent manner to maintain cholesterol homeostasis for Leydig cell steroidogenesis in different conditions. For example, under cholesterol insufficiency, PHB1 may facilitate autophagy / mitophagy to recycle intracellular cholesterol (to maintain steroidogenesis), whereas cholesterol sufficiency and its binding to PHB1 may inhibit PHB1-mediated autophagy / mitophagy. Our finding of changes in the activation levels of an inhibitor of autophagy (i.e., WNK1 kinase) [Gallolu Kankanamalage et al., 2016] in PHB1 manipulated MA-10 cells further support my hypothesis that PHB1's role in Leydig cell steroidogenesis might involve autophagy or related mitophagy. Moreover, as PHB1 plays an important role in Leydig cell steroidogenesis, the loss of mitochondrial PHB1 due to autophagy / mitophagy may be compensated by upregulation of PHB1 under cholesterol deficiency because PHB1 is a cholesterol sensitive gene and is upregulated under cholesterol deficiency [Dong et al., 2010]. Moreover, emerging evidence suggests that lipophagy in Leydig cell plays a role in cholesterol homeostasis

and testosterone production [Ma et al., 2018] and in lipid homeostasis in other cell types [Liu and Czaja, 2013]. The sign of lipophagy as observed in our TEM analysis of the testis samples from the PHB1-Tg mice and in PHB1-manipulated MA-10 cells would mean that PHB1's role in testosterone production might in part be mediated through autophagy, mitophagy, or lipophagy-related cholesterol homeostasis. In addition, lipophagy may protect Leydig cells from the toxic effects of increased lipid accumulation on mitochondrial functions. Thus, PHB1 may function in multiple ways in Leydig cell biology in a context-dependent manner, as observed in the regulation of pERK1/2 levels. The implications of our research findings are broad, particularly in relation to cholesterol homeostasis in steroidogenic cells and mitochondria, as well as in the regulation of autophagy, lipophagy, and mitophagy in steroidogenic cells, adipocytes, cardiomyocytes and hepatocytes.

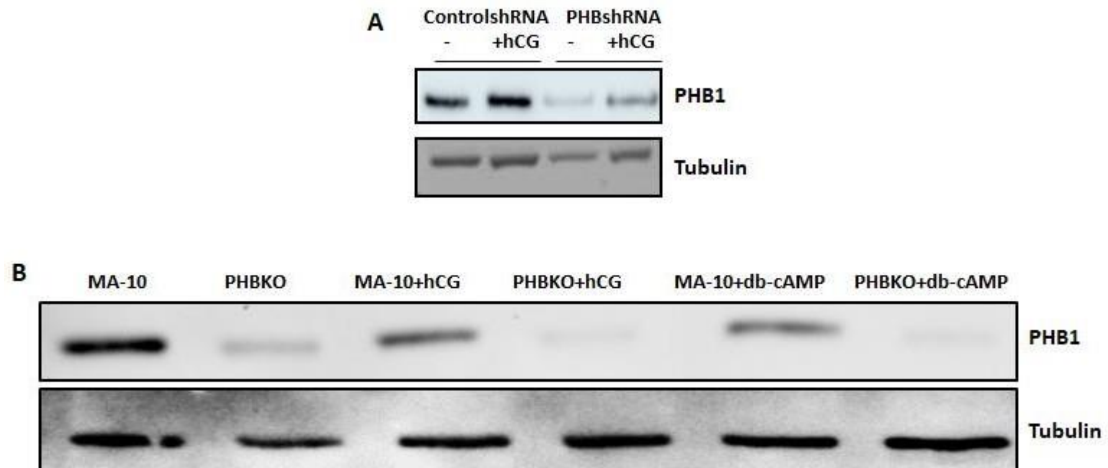
The role StAR plays in the OMM, and the role P450scc plays in the IMM, in the initiation of Leydig cell steroidogenesis is well-established. However, our knowledge of factors involved in their functional coupling across the outer and inner mitochondrial membranes remain limited, including mechanisms involved in cholesterol transport by StAR and its delivery to P450scc. My co-immunoprecipitation data suggest that the mitochondrial heterodimeric complex of PHBs interacts with StAR and P450scc, and PHB1 knockdown inhibits cholesterol transport/homeostasis and P4 production in MA-10 cells. Together, this finding (along with the identification of functional cholesterol binding motifs) suggests that PHB1 plays a role in the functional coupling of StAR and P450scc during steroidogenesis in Leydig cells.

StAR protein levels are known to be acutely regulated in steroidogenic cells in response to trophic hormones [Bose et al., 2002], which remain unclear. An inverse relationship between StAR and PHB1 protein levels in PHB1 manipulated Leydig cells indicate that PHB1 may be related to cellular mechanisms involved in the acute regulation of StAR. In this context, it is important to note that PHB1 interacts with m-AAA and other mitochondrial proteases [Steglich et al., 1999 and Anderson et al., 2020], which may be involved in this relationship. My finding of co-immunoprecipitation of mitochondrial proteases LonP1 and YME-1L1 with PHBs in MA-10 cells support this possibility. However, unlike StAR, the basal PHB1 levels remain maintained in the absence of hCG or db-cAMP stimulation, which make sense in the light of the mitochondrial housekeeping functions of PHB1. To the best of my knowledge, such an acute regulation of PHB1

protein levels has not been reported in any cell type. It would be interesting to know whether PHB1 is regulated similarly in other steroidogenic cell types.

It is interesting to learn that a ubiquitous mitochondrial protein with protein and lipid scaffold properties interacts with cell type-specific proteins (e.g., StAR and P450scc) and produces cell type-specific functions. This finding is consistent with the hypothesis that the mitochondrial attributes of PHB1 contribute to the cell type-specific functions of PHB1 (in addition to its mitochondrial housekeeping functions) [Ande et al., 2016] and further supports my conclusion that PHB1 plays a multifaceted regulatory role in Leydig cell steroidogenesis. In addition, my findings provide a tip-off on how a single protein may function at different levels in cell biology, from a cell-neutral mitochondrial housekeeping function to the cell type-specific functions of mitochondria. Thus, this demonstrates an additional means of creating diversity (due to cellular and functional compartmentalization) from a relatively limited number of genes and proteins, including but not limited to RNA splicing, PTMs, and protein domains.

The scope of our research findings is broad, and may have implications beyond Leydig cell steroidogenesis, such as with regards to corticosteroids and ovarian steroid production. Moreover, the potential health benefits and applications of our findings are wide-ranging. For instance, this new knowledge could possibly be utilized for the development of therapies to treat steroid hormone abnormalities and related comorbidities, and in improving the health of elderly patients by slowing down the decline in muscle and bone health with aging in men, as well as changes in fat distribution (post menopause) and the associated health risks in women. It is likely that these forays will stimulate further investigations to unravel the vital role of PHB1 in steroidogenesis and would lead to important clinical implications.



Supplementary Figure 1. Immunoblots showing the shRNA- and CRISPR/Cas9-mediated knowdown of PHB1 in MA-10 cells

(A) Immunoblots showing the expression levels of PHB1 in MA-10 cells transfected with scramble control shRNA and PHB1-shRNA for 36h.

(B) Immunoblots showing the expression levels of PHB1 in CRISPR/Cas9-mediated Phb knockdown in MA-10 cells

Tubulin is shown as a loading control. All experiments were repeated for at least 3 times.

BRIDGE TO CHAPTER 5

In chapter 4, the role of PHB-1 in testosterone production using transgenic mice testis and MA-10 a Leydig cell line was investigated. In the following chapter (5), I explored the role of intracellular cholesterol pool in regulating cell intrinisic events and factors involved in steroidogenesis.

CHAPTER 5

Exploring a Potential Role of the Intracellular Cholesterol Pool in Steroidogenic Cells

This section has been published as a research article titled:

"The Intracellular Cholesterol Pool in Steroidogenic Cells Plays a Role in Basal Steroidogenesis"

Geetika Bassi, Simarjit Kaur Sidhu, Suresh Mishra

The Journal of Steroid Biochemistry and Molecular Biology 2022. PMID: 35339650
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Highlights

- Cholesterol is more than a precursor substrate for steroidogenesis.
- Cholesterol plays a multifaceted role in basal steroidogenesis.
- The role of cholesterol is conserved across steroidogenic cells.

5.0 Abstract

The framework of steroidogenesis across steroidogenic cells is constructed around cholesterol — the precursor substrate molecule for all steroid hormones — including its cellular uptake, storage in intracellular lipid droplets, mobilization upon steroidogenic stimulation, and finally, its transport to the mitochondria, where steroidogenesis begins. Thus, cholesterol and the mitochondria are highly interconnected in steroidogenic cells. Moreover, accruing evidence suggests that autophagy and mitochondrial dynamics are important cellular events in the regulation of trophic hormone-induced cholesterol homeostasis and steroidogenesis. However, a potential role of cholesterol in itself in the regulation of steroidogenic factors and events remain largely unexplored. We tested the hypothesis that cholesterol plays a role in the regulation of cell-intrinsic factors and events involving steroidogenesis. Here, we show that depleting the intracellular cholesterol pool in steroidogenic cells induces autophagy, affects mitochondrial dynamics, and upregulates steroidogenic factors and basal steroidogenesis in three different steroidogenic cell types producing different steroid hormones. Notably, the cholesterol insufficiency-induced changes in different steroidogenic cell types occur independent of pertinent hormone stimulation and work in a dynamic and temporal manner with or without hormonal stimulation. Such effects of cholesterol deprivation on autophagy and mitochondrial dynamics were not observed in the non-steroidogenic cells, indicating that cholesterol insufficiency-induced changes in steroidogenic cells are specific to steroidogenesis. Thus, our data suggests a role of cholesterol in steroidogenesis beyond being a mere substrate for steroid hormones. The implications of our findings are broad and offer new insights into trophic hormone-dependent and hormone-independent steroidogenesis during development, as well as in health and disease.

5.1 Introduction

Cholesterol, a highly hydrophobic lipid, is an essential component of all animal cell membranes, including the organelle membranes [Elustondo et al., 2017]. Cholesterol critically influences membranes' biophysical and biochemical properties, and consequently, cellular functions at large [Elustondo et al., 2017]. Interestingly, cholesterol distribution is highly heterogeneous amongst subcellular membranes. For example, the cholesterol content of the plasma membrane and the Golgi body membrane are many times higher than that of the endoplasmic reticulum (ER) and the mitochondrial membranes [Montero et al., 2008 and Issop et al., 2013]. In addition to the membrane biology, cholesterol serves as the precursor substrate for all steroid hormones — a major class of regulatory molecules in the body physiology [Issop et al., 2013]. Steroid hormones are produced from cholesterol though a cascade of enzymatic steps called steroidogenesis, which begins in response to the trophic hormone (i.e., ACTH, LH/hCG and FSH) stimulation of their cognate G-protein coupled receptors (GPCRs) in a cell type-specific manner [Azhar et al., 2020]. Notably, steroidogenic enzymes are compartmentalized in the inner mitochondrial membrane (IMM) and the smooth ER membrane, which are relatively very poor in cholesterol content [Elustondo et al., 2017 and Papadopouloa et al., 2012]. This makes cholesterol transport to the IMM (where steroidogenesis begins) a committed step in steroidogenesis [Miller and Bose, 2011], which is dissimilar from other metabolic pathways, where in general, an enzymatic step constitutes the committed step (e.g., *de novo* cholesterol biosynthesis, glycolysis) [Webb et al., 2015 and Amin et al., 1997]. It is conceivable that this special arrangement for steroidogenesis pertaining to cholesterol handling and utilization in steroidogenic cells is essential in fine-tuning their requirement for cholesterol and in executing cell type-specific function without endangering the basic cellular need of cholesterol. Consequently, the steroidogenic cells have evolved precise ways for cholesterol handling and the framework of steroidogenesis is set up around cholesterol, including its cellular uptake, storage in intracellular lipid droplets, and mobilization from lipid droplets upon steroidogenic stimulation, and finally transport to the mitochondria for the initiation of steroidogenesis. For example, steroidogenic acute regulatory (StAR) protein, which is primarily expressed in steroidogenic cells, plays a crucial role in trophic hormone-induced steroidogenesis in the gonads and in the adrenals by facilitating cholesterol transport across outer mitochondrial membrane (OMM) [Stocco, 2000]. Whereas syncytial trophoblast in placenta, which do not express StAR, this essential step in steroidogenesis is

performed by StAR related lipid transfer domain containing 3 (STARD3), also known as metastatic lymph node 64 (MLN64) protein [Tuckey et al., 2004]. This would imply that a dual need of cholesterol by steroidogenic cells is plausibly regulated independently at large (i.e., with or without steroidogenic hormonal stimuli) and the intracellular steroidogenic cholesterol pool itself might play a role in steroidogenesis. Moreover, the GPCRs that mediate the effects of trophic hormones in steroidogenesis might possess a unique relationship with cholesterol (in the presence and absence of respective hormone ligands) because of their cholesterol-centric, cell type-specific functions. However, our understanding of these fundamental aspects of steroidogenesis remains limited. Furthermore, accruing evidence suggests that autophagy — a cell-intrinsic homeostatic process — plays a role in trophic hormone-induced cholesterol uptake and homeostasis in steroidogenic cells [Gao et al., 2018; Li et al., 2011; Tang et al., 1999; Tang et al., 1988 and Gawriluk et al., 2014], and that changes in mitochondrial dynamics are integral to steroidogenesis [Castillo et al., 2015 and Witzig et al., 2020]. Despite the cholesterol-centric arrangement of steroidogenic cells, whether cholesterol in itself plays a role in the regulation of steroidogenic factors and events is very limited and largely unexplored. In 1989, a study by the group of Peter Hall [Iida et al., 1989] examined the effect of altering the ratio of cholesterol to phospholipid levels (by adding exogenous phospholipids) on steroidogenesis in murine Y-1 cells, a model adrenocortical cell line. However, an effect of direct manipulation of cholesterol levels on steroidogenesis, especially after cholesterol depletion was not explored. In addition, the precise relationship between trophic hormone-induced steroidogenic events under a varying intracellular cholesterol pool (e.g., sufficiency and insufficiency) is largely unknown. It is likely that the trophic hormone-induced cell-intrinsic steroidogenic events might vary substantially depending on the available intracellular pool of steroidogenic cholesterol. In other words, a pre-existing cholesterol pool might modify trophic hormone-induced steroidogenic events, and thus, both could modulate each other in a steroidogenic cell type-specific manner. Moreover, the intracellular cholesterol pool might play a role in basal steroidogenesis, which is largely unexplored. This gap in knowledge is likely due to inherent challenges associated with it because of the integral nature of cholesterol in steroidogenesis, as well as being a critical molecule for cellular membranes. For example, perturbing the cholesterol availability using genetic and chemical approaches may not be helpful in deciphering the steroidogenesis-specific function of cholesterol because of their likely interference with the basic cellular need and function of cholesterol. Moreover, blocking

cholesterol transport to the mitochondria or its subsequent utilization for steroidogenesis has its own challenges and pathological consequences, which are well-documented, including within the *StAR* and *Cyp11A1* knockout mouse models [Ishii et al., 2002 and Chien et al., 2013] and in the loss of function mutations in the *StAR* gene in humans [Bose et al., 1996 and Miller et al., 1997]. To overcome these challenges, we used an intermediary approach to investigate the role of cholesterol in the regulation of steroidogenesis, and report that the intracellular steroidogenic cholesterol pool regulates steroidogenic factors and events independent of trophic hormones and plays an important role in basal steroidogenesis. The implications of our findings are broad as they offer new insights into trophic hormone-dependent and hormone independent steroidogenesis during development, as well as in health and disease.

5.2 Materials and Methods

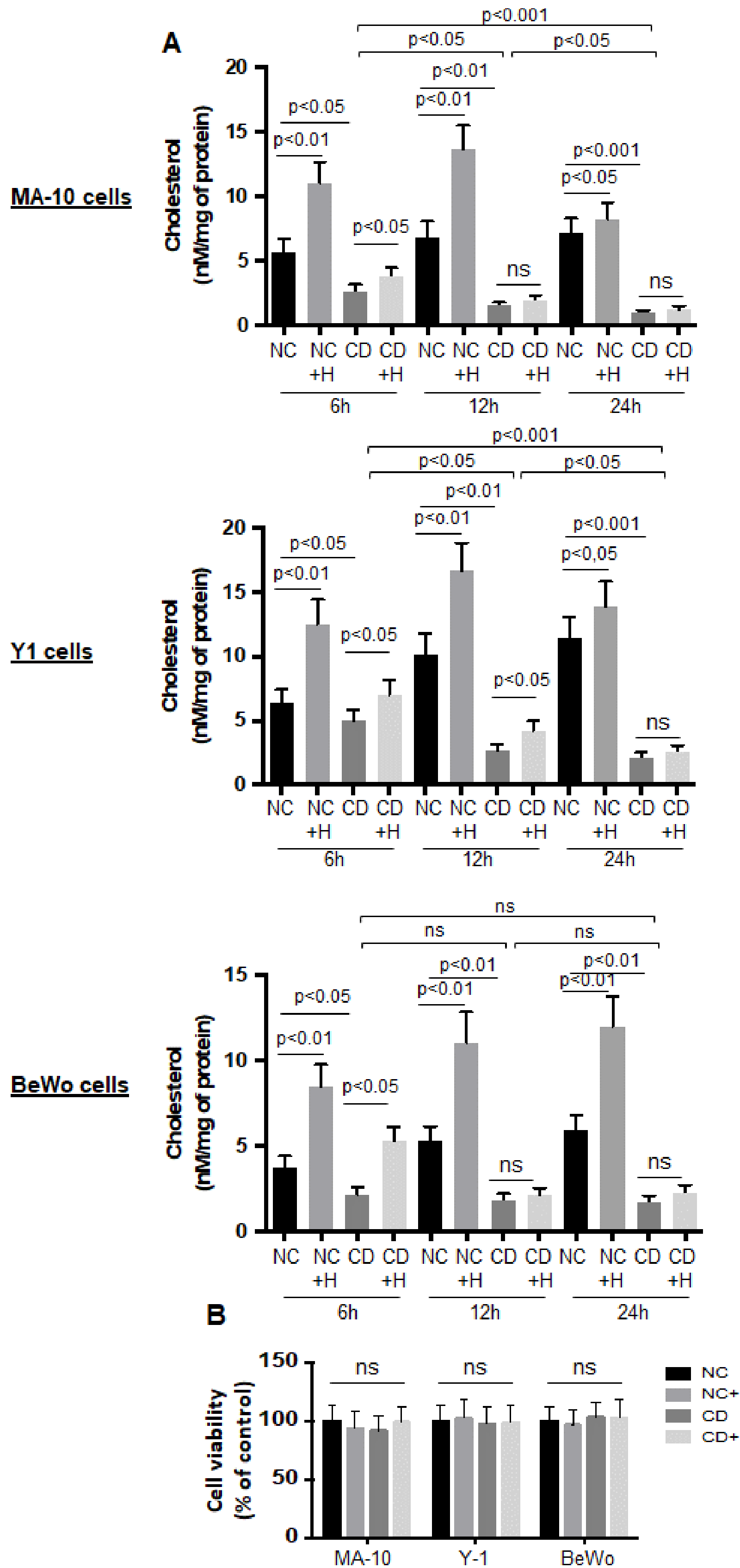
Materials used in Chapter 5 are described in Chapter 3 under section: Chemicals and reagents key resource table. Methods used in this chapter are described in Chapter 3 under the sections: cell culture (3.2.8), cell treatement (3.2.9) depletion of intracellular cholesterol pool in steroidogenic cells (3.2.10), cell transfections (3.2.11), MTT reduction assay (3.2.13), western blotting (3.2.14), transmission electron microscopy (TEM) (3.2.5), amplex™ red cholesterol assay (3.2.17), measurement of pregnenolone level (3.2.19), statstical analysis (3.2.20).

5.3 Results

5.3.1 Culturing steroidogenic cells in a lipoprotein-depleted medium leads to the depletion of the intracellular cholesterol pool & lipid droplets

As cholesterol is the precursor substrate for all steroid hormones, the steroidogenesis related cellular events in steroidogenic cells must respond to intracellular cholesterol insufficiency to maintain basal hormone production, and to meet the physiological demands of different steroid hormones upon trophic hormone stimulation, which vary substantially in different steroidogenic cell types. In this context, the roles of different trophic hormones in the regulation of steroidogenic events and factors are well-established [Azhar et al., 2020]. However, the potential role that cholesterol plays in these events especially under basal conditions is largely unexplored. As cholesterol uptake, autophagy, and mitochondrial dynamics are interlinked to maintain cholesterol

homeostasis and steroidogenesis [Gao et al., 2018; Gawriluk et al., 2014 and Witzig et al., 2020], we hypothesize that the depletion of the intracellular cholesterol pool must affect autophagy and mitochondrial dynamics in steroidogenic cells as a compensatory mechanism to maintain basal steroidogenesis. To explore this, we cultured MA-10 cells (a model murine Leydig cell line) in cholesterol-depleted (CD) cell culture conditions (i.e., culture medium supplemented with lipoprotein depleted FBS containing only 0.04 mg/ml cholesterol — an approximately 35-fold reduction compared with normal FBS, which contains around 1.40 mg/ml cholesterol) for different time points (i.e., 6h, 12h and 24h). To confirm intracellular cholesterol depletion under the experimental condition used, cells were harvested at each time point to determine their cholesterol contents. Under unstimulated condition, a gradual decrease in cholesterol content was observed at 6h, 12h and 24h in MA-10 cells grown in the CD medium (Fig. 5.1A). Moreover, at each time point, cholesterol contents in CD group were significantly lower than the cholesterol contents of cells cultured in the normal culture (NC) condition (i.e., the culture medium supplemented with the normal FBS) (Fig. 5.1A). A similar effect was found in Y-1 cells (a model murine adrenocortical cell line) between different time points within CD group and between NC and CD groups at each time point (Fig. 5.1A). However, such differences in cholesterol contents between 12h and 24h were not observed in BeWo cells (a model human choriocarcinoma cell line) in CD group (Fig. 5.1A). However, differences between NC and CD group in BeWo cells were similar to MA-10 and Y-1 cells (Fig. 5.1A). Under stimulated condition, the CD group showed both similarities and differences in their response in MA-10, Y-1 and BeWo cells. For example, in all three-cell lines, a significant difference between unstimulated and stimulated cells was observed only at 6h, whereas in Y-1 cells such difference was also found at 12h (Fig. 5.1A), which was not found in MA-10 and BeWo cells. Whereas in the NC experimental group, significant differences were observed between unstimulated and stimulated cells at all three time points (Fig. 5.1A). No significant change in cell viability were observed during the 24h under the CD culture conditions used when compared with cells grown in NC culture conditions (Fig. 5.1B).



A
MA-10 cells
Cholesterol (nM/mg of protein)
p<0.001
p<0.05
p<0.05
p<0.01
p<0.01
p<0.05
p<0.01
p<0.001
p<0.05
p<0.05
ns
ns
NC NC +H CD CD +H NC NC +H CD CD +H NC NC +H CD CD +H
6h 12h 24h
Y1 cells
Cholesterol (nM/mg of protein)
p<0.001
p<0.05
p<0.05
p<0.01
p<0.01
p<0.05
p<0.01
p<0.001
p<0,05
p<0.05
p<0.05
ns
BeWo cells
Cholesterol (nM/mg of protein)
ns
ns
ns
p<0.01
p<0.01
p<0.05
p<0.01
p<0.01
p<0.01
p<0.05
ns
ns
B
Cell viability (% of control)
ns
ns
ns
MA-10 Y-1 BeWo
NC
NC+
CD
CD+

Figure 5.1. Culturing steroidogenic cells in lipoprotein-depleted medium leads to depletion of intracellular cholesterol pool.

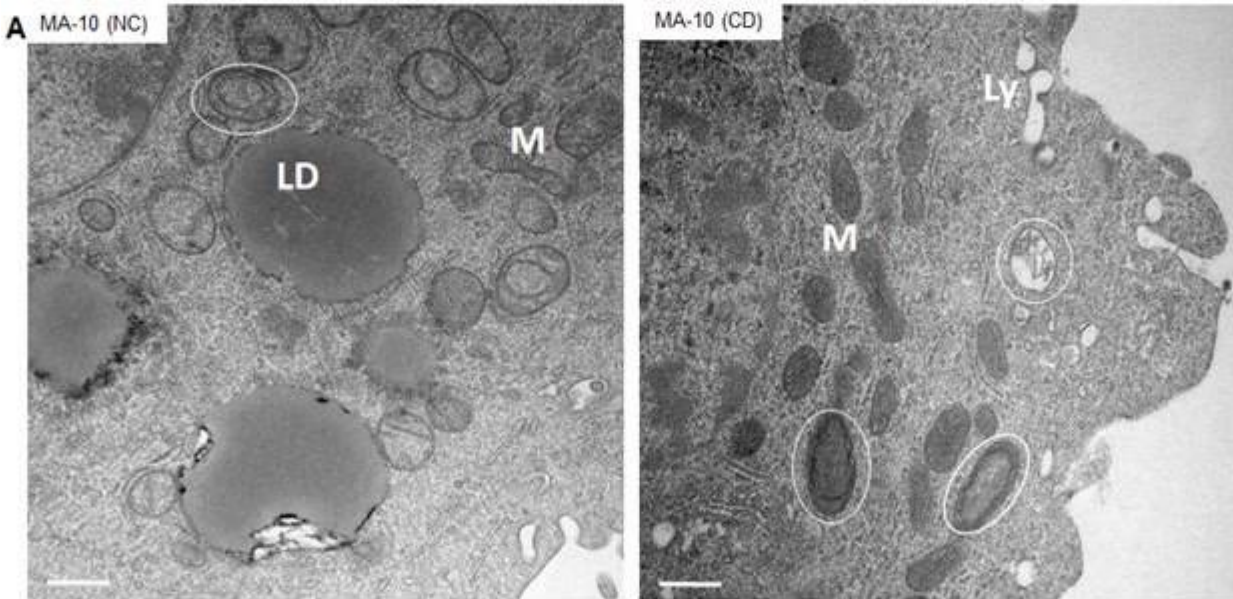
(A) Histograms showing cholesterol levels in different steroidogenic cell types as determined Amplex^{TM} Red Assay. NC – normal culture condition, CD – cholesterol depleted culture condition, H – hormone treatment (hCG or ACTH, as applicable), ns – not significant. Data are presented as mean ± SEM (n = 3).

(B) Histograms showing cell proliferation of different steroidogenic cell types under NC and CD culture conditions as determined by MTT assay. Data are presented as percentage of control (mean ± SEM, n = 3). ns – not significant.

5.3.2 Ultrastructural analysis of steroidogenic cells after the manipulation of the intracellular cholesterol pool

We also confirmed the depletion of intracellular lipid droplets and the cholesterol pool in MA-10, Y-1 and BeWo cells cultured in the CD medium using transmission electron microscopy (TEM) (Figs. 5.2-5.4). Lipid droplets were apparent in the MA-10 and Y1 cells in the NC experimental group, which substantially reduced in size or almost disappeared in the CD experimental group (Figs. 5.2, 5.3). In the BeWo cells (a model human trophoblast cell line), mitochondria and lipid droplets were found to be relatively poor (in number and size) than the MA-10 and Y-1 cells. In addition, the signs of autophagy, such as pre-autophagosomes, autophagosomes, and autolysosomes, were in all three cell lines, which were more noticeable in CD experimental group (Figs. 5.2-5.4).

Importantly, an increase in lysosome size and numbers were consistently found in cells under CD conditions, which inversely correlated with the depletion of lipid droplets and the cholesterol pool, suggesting a compensatory increase in cholesterol mobilization. This data established that culturing the MA-10, Y-1 and BeWo steroidogenic cells in the CD medium leads to the depletion of intracellular cholesterol content without affecting cell viability under the culture conditions used.



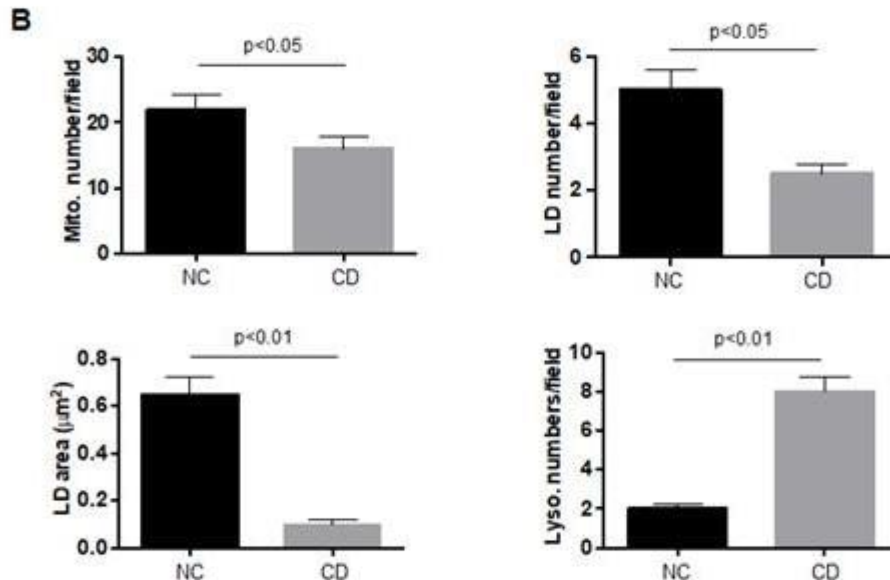
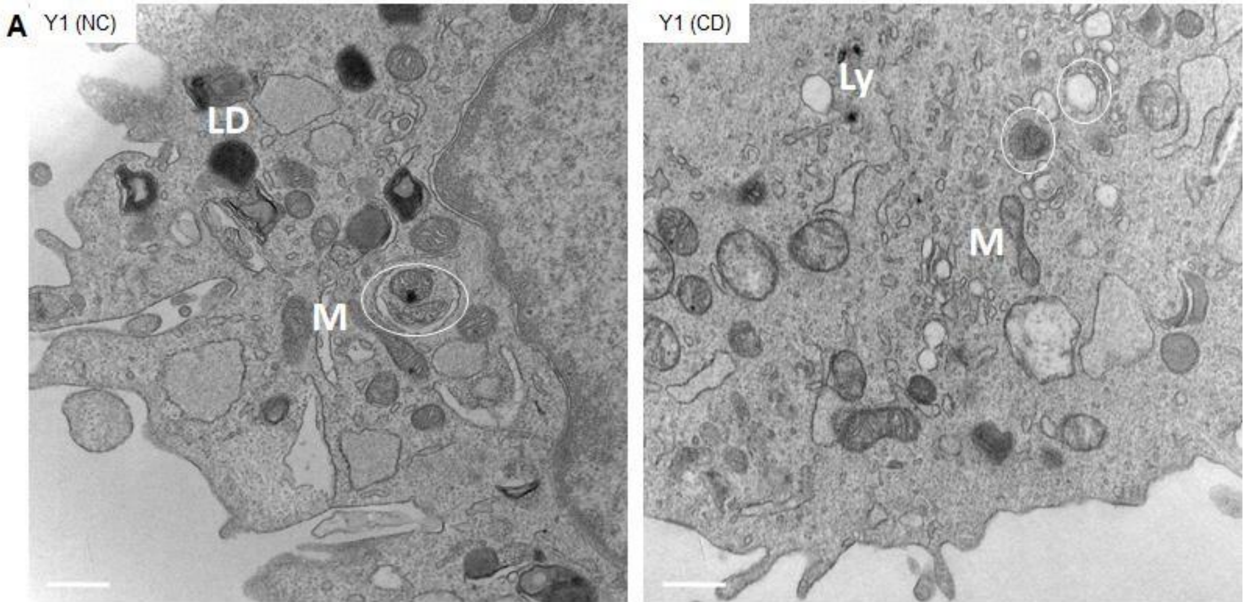


Figure 5.2. TEM analysis of MA-10 cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition.

(A) Representative transmission electron micrographs (magnification: 50000x) of MA-10 cells cultured under cholesterol deficient and normal culture conditions. Scale bars = 500 nm. LD – lipid droplets, Ly – lysosomes, M – mitochondria. Circle – signs of autophagy/lipophagy, NC – normal culture condition, CD – cholesterol depleted culture condition.

(B) Histograms showing quantification of LDs size, as well as lysosomal and mitochondrial numbers. Data are presented as mean ± SEM (n = 3).



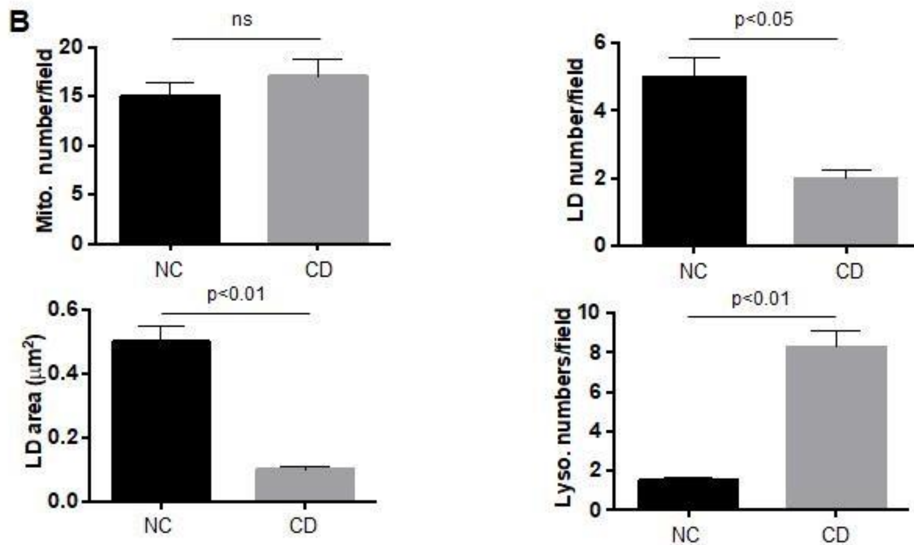
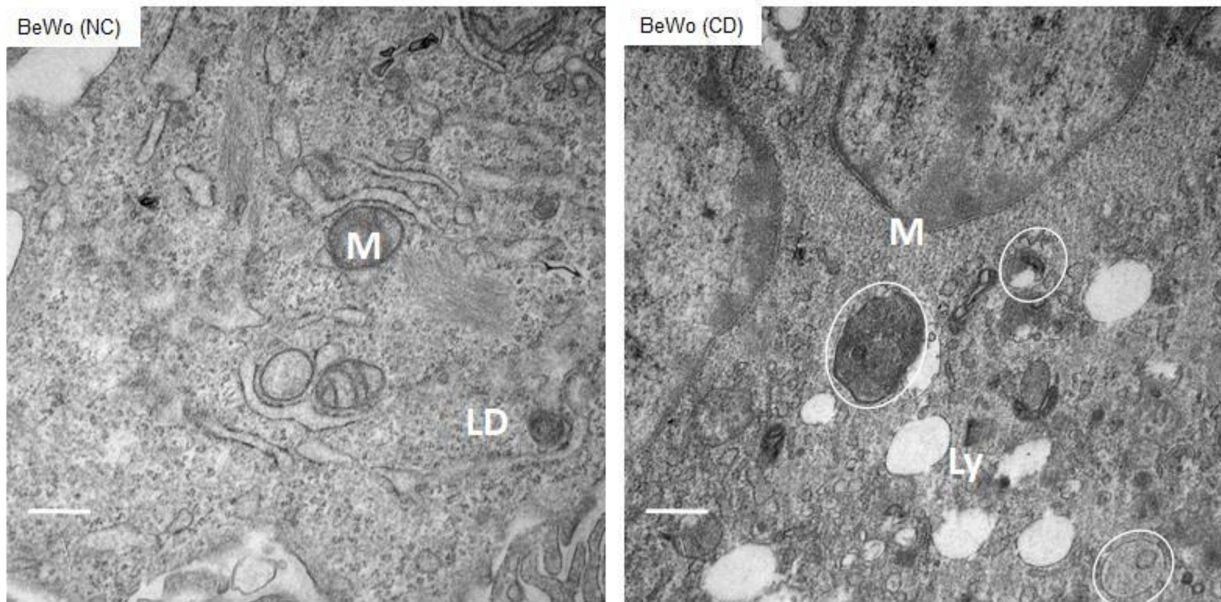


Figure 5.3. TEM analysis of Y-1 cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition.

(A) Representative transmission electron micrographs (magnification: 50000x) of Y-1 cells cultured under cholesterol deficient and normal culture conditions. Scale bars = 500 nm. LD – lipid droplets, Ly – lysosomes, M – mitochondria. Circle – signs of autophagy/lipophagy, NC – normal culture condition, CD – cholesterol depleted culture condition.

(B) Histograms showing quantification of LDs size, as well as lysosomal and mitochondrial numbers. Data are presented as mean ± SEM (n = 3).



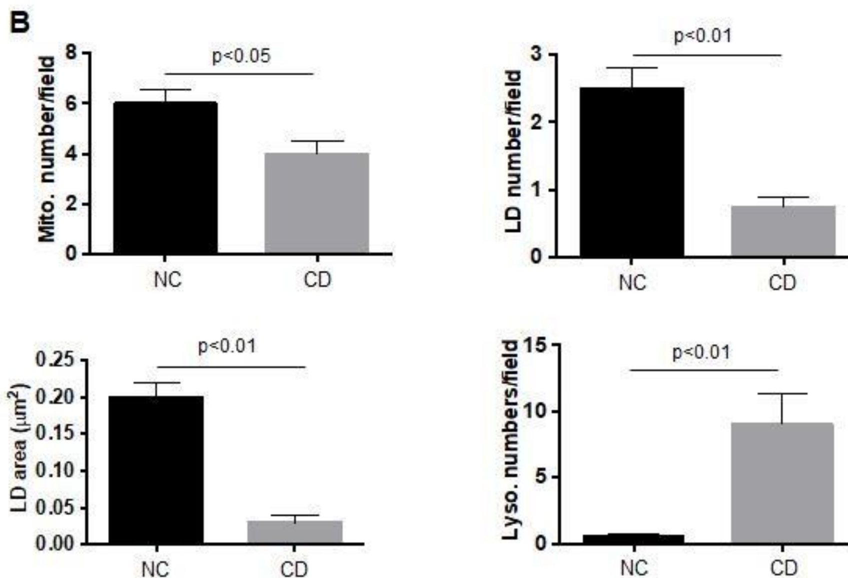


Figure 5.4. TEM analysis of BeWo cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition.

(A) Representative transmission electron micrographs (magnification: 50000x) of different steroidogenic cells cultured under cholesterol deficient and normal culture conditions. Scale bars = 500 nm. LD – lipid droplets, Ly – lysosomes, M – mitochondria. Circle – signs of autophagy/lipophagy, NC – normal culture condition, CD – cholesterol depleted culture condition. **(B)** Histograms showing quantification of LDs size, as well as lysosomal and mitochondrial numbers. Data are presented as mean ± SEM (n = 3).

5.3.3 Cholesterol deprivation induces steroidogenic events and factors in Leydig cells

Next, we analyzed cell lysates prepared at each time point for the markers of autophagy, mitochondrial dynamics, and steroidogenesis, including proteins involved in cholesterol handling using immunoblotting. As hypothesized, an increase in the autophagy marker LC3-II was observed in the MA-10 cells in response to CD (independent of steroidogenic stimulation) at 6h and 12h, when compared with respective control groups cultured under NC conditions (Fig. 5.5). No difference in band intensity of LC3 (I and II) was observed between NC and CD groups at 24h. Thus, LC3 II band intensity was highest at 6h, which gradually tappers at 12h and 24h (Fig. 5.5). LC3-I band intensity was also relatively stronger in the CD experimental groups at 6h and 12h compared with their respective control groups (Fig. 5.5). Steroidogenic stimulation further increased LC3-II band intensity at 12h and 24h under CD conditions compared with unstimulated cells (Fig. 5.5); which was apparent at 24h when CD effect almost disappeared. Such changes in the ATG7 levels were not observed in MA-10 cells (Fig. 5.5). Next, we examined the protein levels of mitochondrial fission (DRP1) and fusion markers (OPA1 and MFN2) to find the potential relationship between steroidogenic cholesterol availability and mitochondrial dynamics. At 6h and 12h, DRP1 levels remain unchanged between NC and CD under unstimulated condition (Fig. 5.5). However, at 6h and 24h, a decrease in Drp1 level was observed in the CD group when compared with the NC group under stimulated condition (Fig. 5.5). In general, OPA1 levels showed some similarity to DRP1 at 6h and 12h (under unstimulated condition); however, an opposite effect was observed between DRP1 and OPA1 between NC and CD at 12h under stimulated condition (Fig. 5.5). In addition, a difference in the OPA1 band was noticed in the presence and absence of steroidogenic stimulation at 12h and 24h (Fig. 5.5), which was lower at 12h and higher at 24h under stimulated condition. Similar to OPA1 levels, MFN2 levels were higher in stimulated condition at 6h but remain unchanged at 12h and 24h (Fig. 5.5). Collectively, this evidence suggests a putative link between intracellular cholesterol availability, autophagy, and mitochondrial dynamics in the MA-10 cells, which appears to work differently under basal and stimulated conditions.

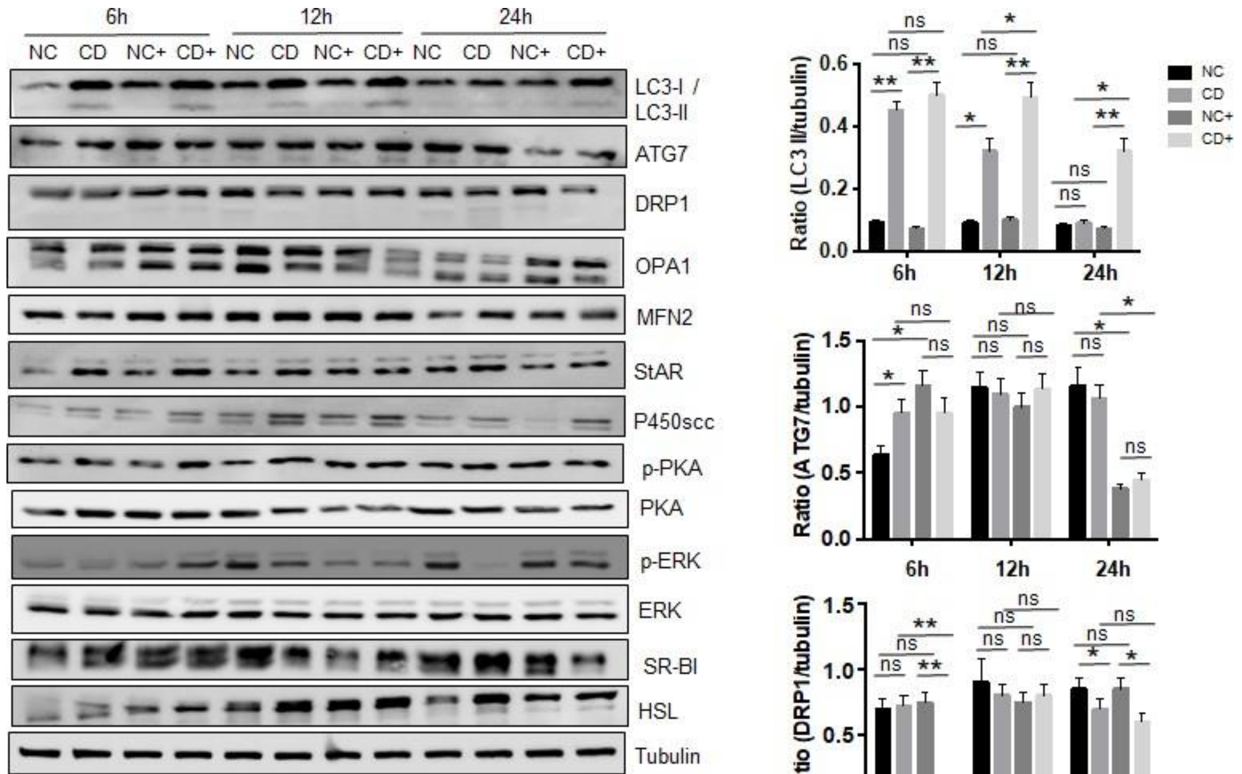
Next, we examined the protein levels of StAR and P450scc because of their roles in cholesterol transport to the mitochondria and subsequent utilization for steroidogenesis, respectively. Interestingly, both protein levels increased in CD experimental group under unstimulated condition at three time points (i.e., 6h and 12h than at 24h) (Fig. 5.5) and showed a

temporal change. Maximum increase in StAR was found at 6h whereas in the case of P450scc, it was 12h (Fig. 5.5). As expected, hormone stimulation led to increase in both protein levels; however, StAR level peaked earlier (at 6h) than P450scc level (at 12h) (Fig. 5.5). Moreover, the effect of cholesterol depletion was also apparent under stimulated condition (Fig. 5.5). This data suggests that cholesterol insufficiency in steroidogenic cells leads to upregulation of proteins involved in steroidogenesis. This finding prompted us to examine any upstream signaling events, because StAR and P450scc, as well as autophagy and mitochondrial dynamics, are known to be regulated by steroidogenic signaling [Miller et al., 2011; Gao et al., 2018; Yi et al., 1999; Tang et al., 1988; Gawriluk et al., 2014; Castillo et al., 2015 and Witzig et al., 2020]. As the cAMP-PKA and MAPK-ERK pathways are major mediators of trophic hormone-induced cell signaling in steroidogenic cells [Lei et al., 2001; Miller et al., 2011 and Seger et al., 2017], including Leydig cells [Seger et al., 2017 and Medar et al., 2021], we examined the phosphorylation levels of PKA and ERK in response to CD. A distinct temporal effect on p-PKA and p-ERK was observed under unstimulated condition.

The p-PKA level was increased under CD at 12h and 24h under both conditions (with or without hormonal stimulation) (Fig. 5.5). Under unstimulated condition, CD led to down regulation of p-ERK1/2 levels at 12h and 24h, which was almost abolished at 24h (Fig. 5.5). Whereas under stimulated state, a diverse and temporal effect was observed on p-ERK1/2 levels in CD group, which was increased at 6h, unchanged at 12h, and reduced at 24h (Fig. 5.5). Together, this data suggests a dynamic relationship between cholesterol availability and p-ERK signaling in MA- 10 cells. For instance, steroidogenic stimulation prevented a CD-induced decrease in p-ERK levels at 6h and 24h but not at 12h (Fig. 5.5).

In addition, we measured the expression levels of proteins involved in cholesterol uptake (SR-B1) and mobilization (HSL) [Duarte et al., 2014 and Bassi et al., 2021] using immunoblotting. No significant difference in SR-B1 levels was observed at 6h and 24h in response to CD compared with NC group (without hormonal stimulation) whereas a reduction was observed at 12h (Fig. 5.5). In the presence of hormone stimulation, an opposite effect was observed between cells cultured under CD and NC at 12h and 24h, which was higher in CD group at 12h and lower at 24h (Fig. 5.5). Notably, the HSL level was found to be progressively increased in CD group under unstimulated condition and remain higher upon steroidogenic stimulation when compared with the respective NC experimental group (Fig. 5.5). This data suggests a relationship between

steroidogenic signaling related to cholesterol uptake and mobilization with preexisting cholesterol pool in the regulation of SR-B1 and HSL levels. In aggregate, our data suggests that steroidogenic cholesterol insufficiency in itself leads to the upregulation of steroidogenic factors and events in MA-10 cells independent of steroidogenic stimulation, suggesting that it plays an important role in basal steroidogenesis.


6h
12h
24h
NC CD NC+ CD+ NC CD NC+ CD+ NC CD NC+ CD+
LC3-I / LC3-II
ATG7
DRP1
OPA1
MFN2
StAR
P450scc
p-PKA
PKA
p-ERK
ERK
SR-BI
HSL
Tubulin
Ratio (LC3 II/tubulin)
Ratio (ATG7/tubulin)
Ratio (DRP1/tubulin)
NC
CD
NC+
CD+

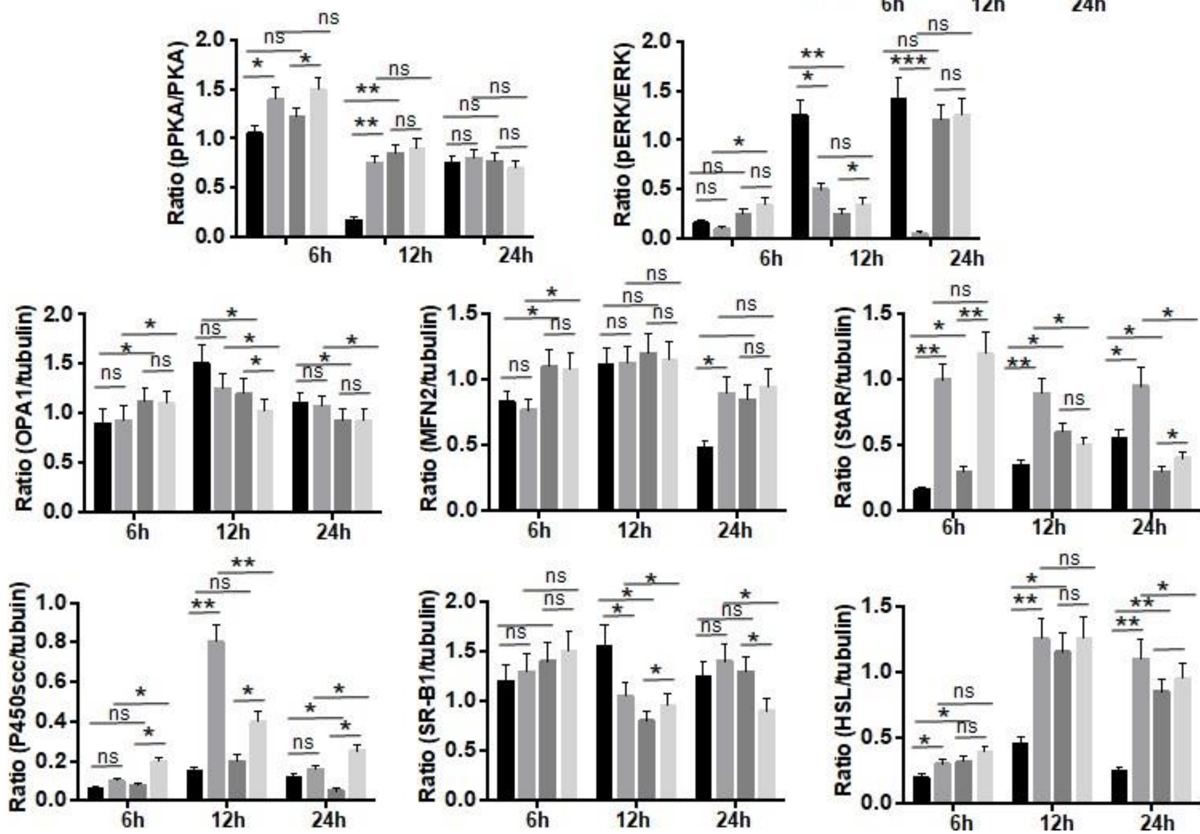

Ratio (pPKA/PKA)
Ratio (pERK/ERK)
Ratio (OPA1/tubulin)
Ratio (MFN2/tubulin)
Ratio (StAR/tubulin)
Ratio (P450scc/tubulin)
Ratio (SR-B1/tubulin)
Ratio (HSL/tubulin)
6h
12h
24h

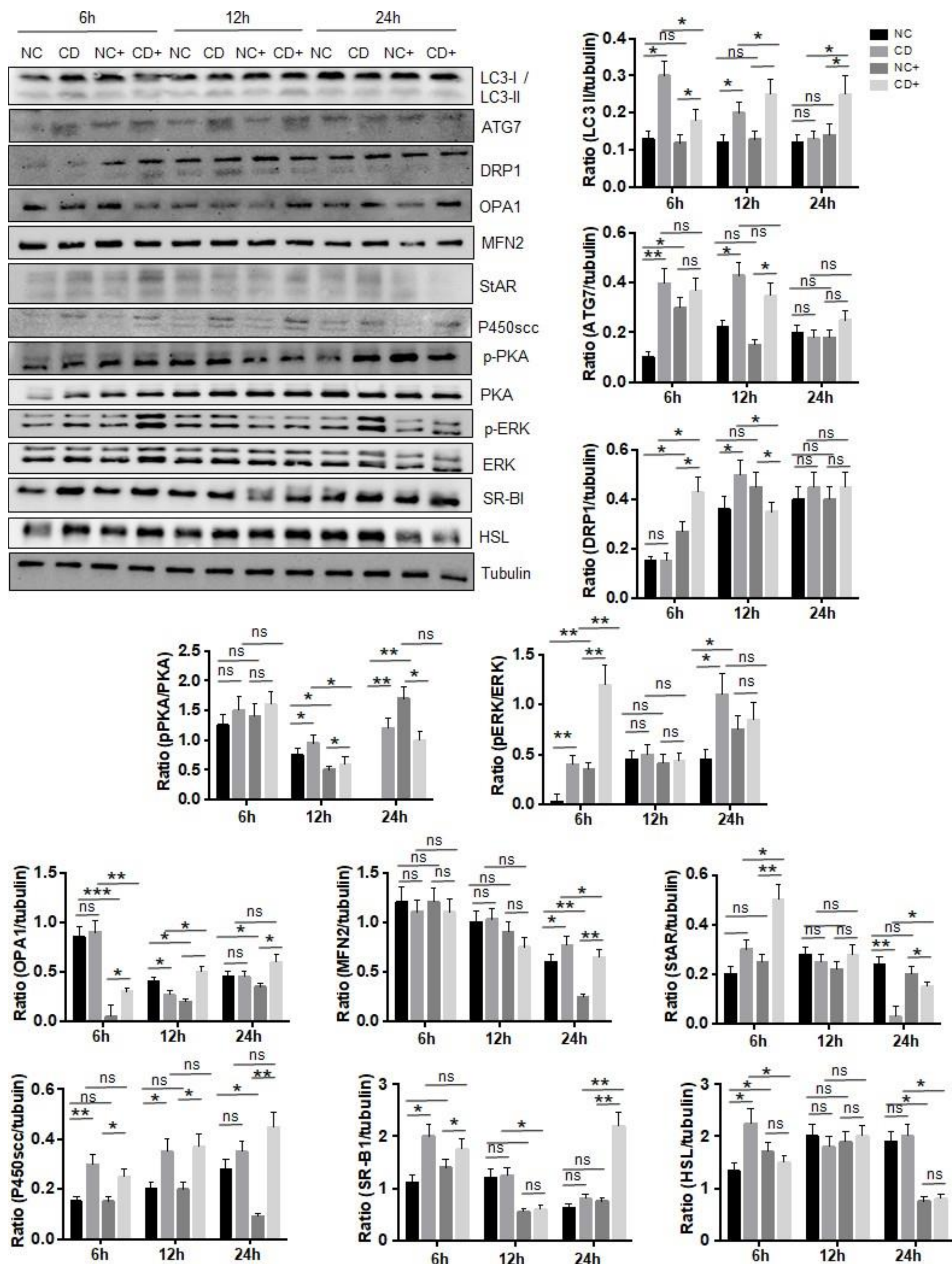
Figure 5.5. Intracellular cholesterol depletion induces steroidogenic factors and events in MA- 10 cells.

Representative immunoblots showing protein levels of autophagy, mitochondrial dynamics, and steroidogenic markers in MA-10 cells cultured under normal and cholesterol depleted conations. Tubulin blot is shown as a loading control. + indicates hormonal stimulation (i.e., hCG), NC – normal culture condition, CD – cholesterol depleted culture condition. All experiments were repeated for at least for three times. Quantification of protein band densities are shown with histograms. Legend in LC3 II histograms is applicable to all histograms. Data are presented as mean ± SEM (n = 3).

5.3.4 The role of cholesterol in basal steroidogenesis is conserved in adrenocortical and placental cells

To determine whether the observed effects of CD on steroidogenic events and factors are MA-10 cell-specific or pertinent to steroidogenic cells, we repeated this experiment in adrenocortical Y-1 cells [Clark et al., 2015] and placental BeWo cells [Wang et al., 2021], as well as in a non-steroidogenic cell (i.e., H9c2 cells, a model rat myocardial cell line). Similar to MA-10 cells, CD resulted in increased LC3-II levels at 6h and 12h (in unstimulated cells) when compared with NC group (Fig. 5.6). Similarly, no difference in band intensity of LC3 (I and II) was observed between NC and CD groups at 24h. Notably in Y-1 cells, the effect of CD on ATG7 was more apparent than MA-10 cells (Figs. 5.5 & 5.6). In addition, the effect of CD on LC3-II and ATG7 was tapered at 24h (Fig. 5.6). Moreover, similar to MA-10 cells, DRP1 levels remain unchanged between NC and CD experimental groups at 6h; however, an increase was observed in CD group at 12h (Fig. 5.6). In general, OPA1 levels showed an opposite in relation to DRP1 during 6h-12h. However, a difference in DRP1 was observed between NC and CD groups at 24h, which were higher in NC group under both unstimulated and stimulated conditions (Fig. 5.6). Moreover, changes in StAR and P450scc levels (under NC and CD conditions) in Y-1 cells showed a pattern similar to MA-10 cells, which were consistently higher in CD experimental group (Fig. 5.6). In general, the higher molecular weight isoform of StAR was more apparent in Y-1 cells whereas in MA-10 cells, it was the lower molecular weight isoform (Fig. 5.5 & 5.6). Notably at 24h, the modulatory effect of CD on p-PKA in Y-1 cells showed an opposite effect than MA-10 under hormonal stimulation. In both cell lines, CD resulted in increase in p-PKA levels compared with NC group (Figs. 5.5, 5.6) under unstimulated state; however, after hormonal stimulation in Y-1 cells, CD appears to have a negative effect on p-PKA suggesting a potential interplay between the two (Fig. 5.6). The p-ERK1/2 levels in Y-1 cells were progressively increased in CD group compared with NC group under unstimulated state (Fig. 5.6). Whereas a varied effect on p-ERK1/2 level was observed in Y-1 cells under hormonal stimulation, ranging from synergistic stimulatory effect at 6h and an inhibitory effect at 24h (Fig. 5.6). In MA-10 cells, SR-BI level was decreased at 12h whereas HSL levels increased at 12h and 24h under CD condition when compared with respective NC group (Fig 5.5). Hormonal stimulation was found to have inhibitory effects on SR-BI and HSL levels at 12h and 24h (Fig 5.5). In Y-1 cells, SR-B1 and HSL levels increased under CD at 6h but not at 12h and 24 h (Fig 5.6). Hormonal stimulation showed inhibitory effect on CD-

induced changes in SR-BI levels at 12h and a stimulatory effect at 24h whereas no effect was observed on HSL levels at 12h (Fig 5.6). Thus, trophic hormone stimulation appears to have a modulatory effect on CD-induced changes in steroidogenic events and factors, suggesting their cross-regulation (Figs. 5.6).



6h
12h
24h
NC
CD
NC+
CD+
LC3-I /
LC3-II
ATG7
DRP1
OPA1
MFN2
StAR
P450scc
p-PKA
PKA
p-ERK
ERK
SR-BI
HSL
Tubulin
Ratio (LC3 II/tubulin)
Ratio (ATG7/tubulin)
Ratio (DRP1/tubulin)
Ratio (pPKA/PKA)
Ratio (pERK/ERK)
Ratio (OPA1/tubulin)
Ratio (MFN2/tubulin)
Ratio (StAR/tubulin)
Ratio (P450scc/tubulin)
Ratio (SR-B1/tubulin)
Ratio (HSL/tubulin)

Figure 5.6. Intracellular cholesterol depletion induces steroidogenic factors and events in Y-1 cells.

Representative immunoblots showing protein levels of autophagy, mitochondrial dynamics, and steroidogenic markers in Y-1 cells cultured under normal and cholesterol depleted conations. Tubulin blot is shown as a loading control. + indicates hormonal stimulation (i.e., ACTH), NC – normal culture condition, CD – cholesterol depleted culture condition. All experiments were repeated at least for three times. Histograms showing quantification of protein band densities. Legend in LC3 II histograms is applicable to all histograms. Data are presented as mean ± SEM (n = 3).

In BeWo cells, basal LC3-II levels were found to be relatively higher than in the MA-10 and Y-1 cells (Fig. 5.7). A significant increase in LC3-II level was observed in CD group at 6h (when compared with NC group); however, no difference was observed at12h, whereas a reduction in LC3-II level was observed at 24h (Fig. 5.7). In NC group, hormonal stimulation increased its levels at 6h and decreased at 12h and 24h (Fig. 5.7). Whereas under CD condition, hormonal stimulation has no effect at 6h and 12h but increased at 24h when compared with their respective unstimulated control group (Fig. 5.7). Unlike MA-10 cells and Y-1 cells, a distinct doublet band of ATG7 was observed in NC group but not in CD group without/with hormonal stimulation (Fig. 5.7). The doublet band density was highest at 6h, which gradually decreased at 12h and 24h (Fig. 5.7). Among markers of mitochondrial dynamics, no difference in OPA1 levels was observed at 6h and 12h; whereas hCG stimulation increased its levels at 12h in cells cultured under NC and CD states (Fig. 5.7). CD appears to have no effect on Opa1 levels. Unlike OPA1, MFN2 showed an increase at 6h under CD with/without hCG stimulation (Fig. 5.7). Moreover, DRP1 level was found to increase in response to hCG at 6h and 12h under CD culture condition, which was not observed under NC culture condition (Fig. 5.7); however, an increase was observed at 24h under the same condition. Among cell signaling intermediates, p-ERK1/2 levels showed an opposite effect in response to hCG stimulation in NC and CD groups at 6h and 12h, which was decreased in the former and increased in the later when compared with their respective control groups (Fig. 5.7). This trend was reversed at 24 (Fig. 5.7). The p-PKA levels showed a varied pattern between NC and CD in response to hCG stimulation at different time points. At 6h, p-PKA levels increased in CD group, which was observed at 12h and 24h (Fig. 5.7). Whereas in NC group, p-PKA levels substantially increased at 24h (Fig. 5.7). Collectively, this data further supports our general findings that the availability of the intracellular cholesterol pool in steroidogenic cells plays a role in the regulation of steroidogenic factors and events in a context-dependent manner.

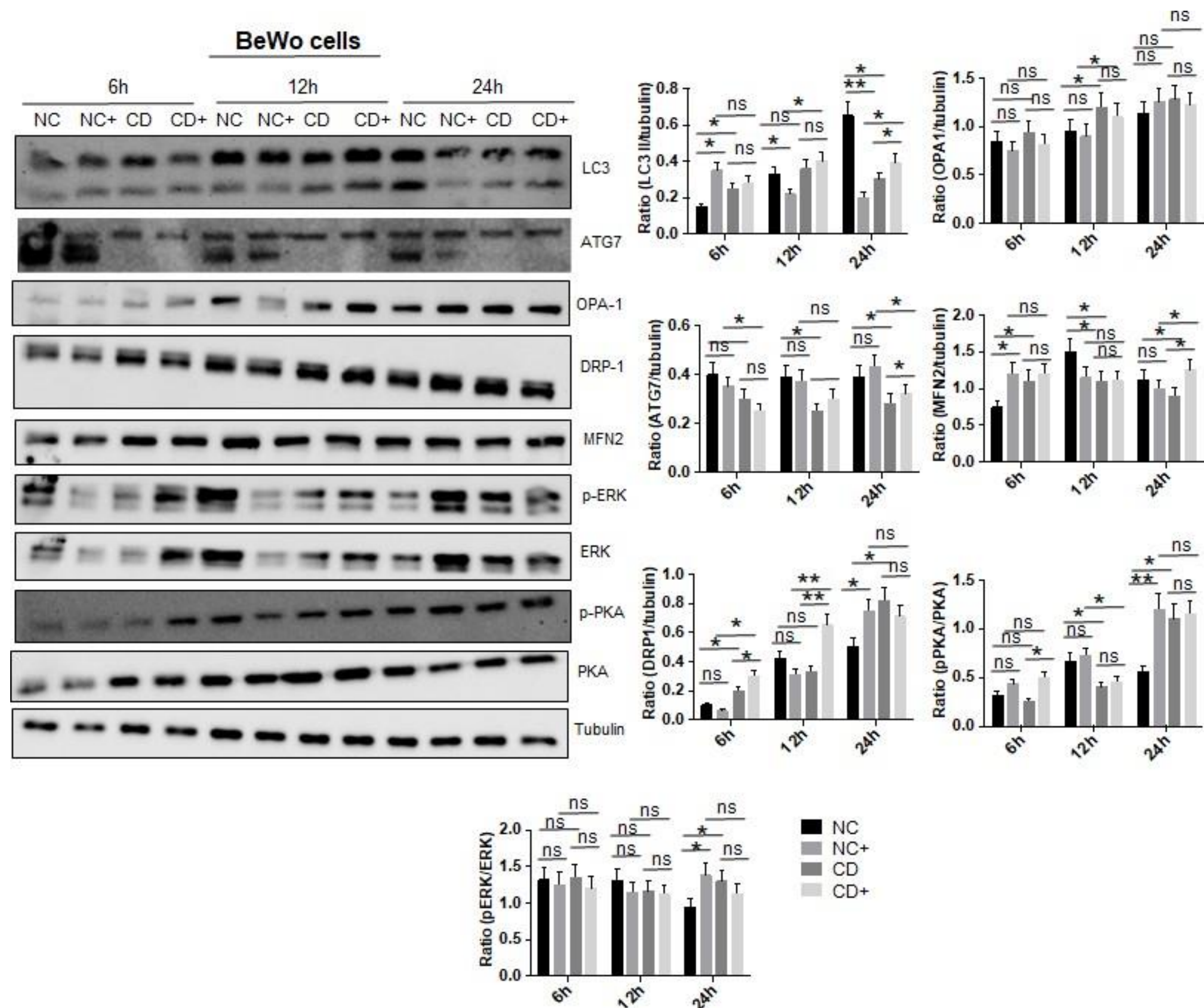


Figure 5.7. Intracellular cholesterol depletion induces steroidogenic factors and events in BeWo cells.

Representative immunoblots showing protein levels of autophagy, mitochondrial dynamics, and steroidogenic markers in steroidogenic BeWo cells cultured under normal and cholesterol depleted conations. Tubulin blot is shown as a loading control. + indicates hormonal stimulation (i.e., hCG), NC – normal culture condition, CD – cholesterol depleted culture condition. All experiments were repeated at least for three times. Histograms showing quantification of protein band densities. Data are presented as mean ± SEM (n = 3).

To determine whether CD-induced changes in the markers of autophagy and mitochondrial dynamics are steroidogenic cell specific or not, I repeated CD experiment using a non-steroidogenic H9c2 cells (which is a model rat myocardial cell line) and analyzed cell lysates using immunoblotting. The basal LC3-II levels were found to be relatively much higher in H9c2 cells than steroidogenic cells, which further increased under CD condition (Fig. 5.8). Among mitochondrial dynamics markers, DRP1 levels showed a decreasing trend during 6-24h whereas MFN2 levels displayed an increasing trend during 6-24h (Fig. 5.8). Thus, both markers showed a distinct pattern than steroidogenic cells indicating that CD-induced changes in autophagy and mitochondrial dynamics markers in steroidogenic cells appear to be cell type-specific.

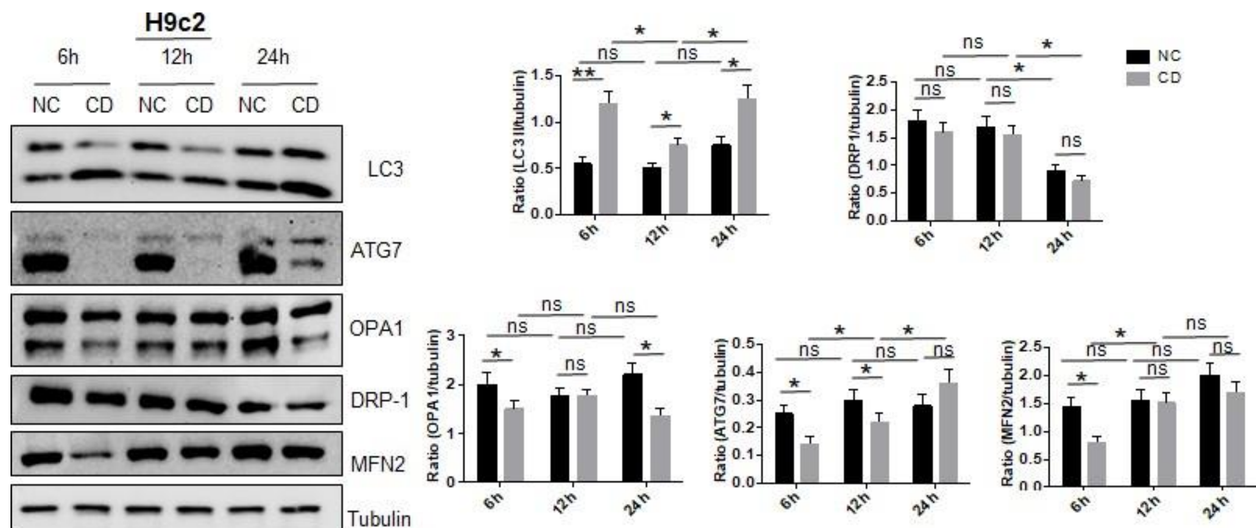


Figure 5.8. Intracellular cholesterol depletion induced in non-steroidogenic H9c2 cells. Representative immunoblots showing protein levels of autophagy, mitochondrial dynamics, and steroidogenic markers in non-steroidogenic H9c2 cells cultured under normal and cholesterol depleted conations. Tubulin blot is shown as a loading control. NC – normal culture condition, CD – cholesterol depleted culture condition. All experiments were repeated at least for three times. Histograms showing quantification of protein band densities. Data are presented as mean ± SEM (n = 3).

In addition to depleting the intracellular cholesterol pool, we investigated the effect of an increased intracellular cholesterol pool on ultrastructural changes in the MA-10 cells and Y-1 cells. For this, we used a shRNA-mediated knockdown of *StAR* as a model, because in both adrenocortical and Leydig cell types, the StAR protein is essential for cholesterol transport to the mitochondria for steroidogenesis [Papadopoulos et al., 2012 and Miller et al., 2011], and loss of StAR function is known to cause lipid and cholesterol accumulation in these cell types [Bose et al., 1996 and Miller et al., 1997]. As expected, the *StAR* knockdown in the MA- 10 and Y-1 cells led to an increase in lipid droplets and a decrease in mitochondrial numbers (only in Y-1 cells), as revealed by TEM analysis (Fig. 5.9). In addition, signs of mitochondrial damage, including smaller and fragmented mitochondria, as well as disorganization and fragmentation of mitochondrial cristae were observed (Fig. 5.9). However, a difference in LDs degradation was apparent in StAR knockdown MA-10 and Y-1, which was apparent in MA-10 cells whereas in Y-1cells, early signs of lipophagy (e.g., pre-lipophagosomes) were noticeable, such as appearance of lipophagic membrane (Fig. 5.9).

MA-10 (sc)
M
LD

MA-10 (sh)
M
Ly
LD

Y1 (sc)
M
LD

Y1 (sh)
LD
M

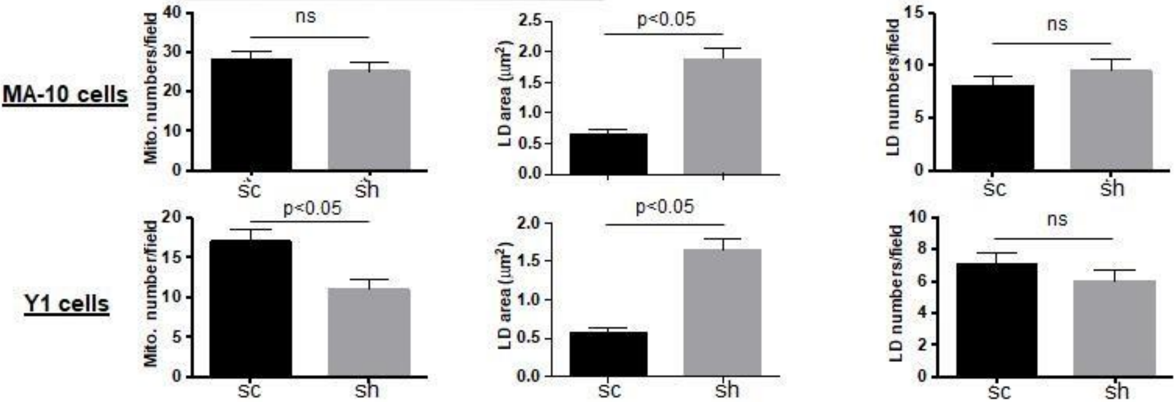
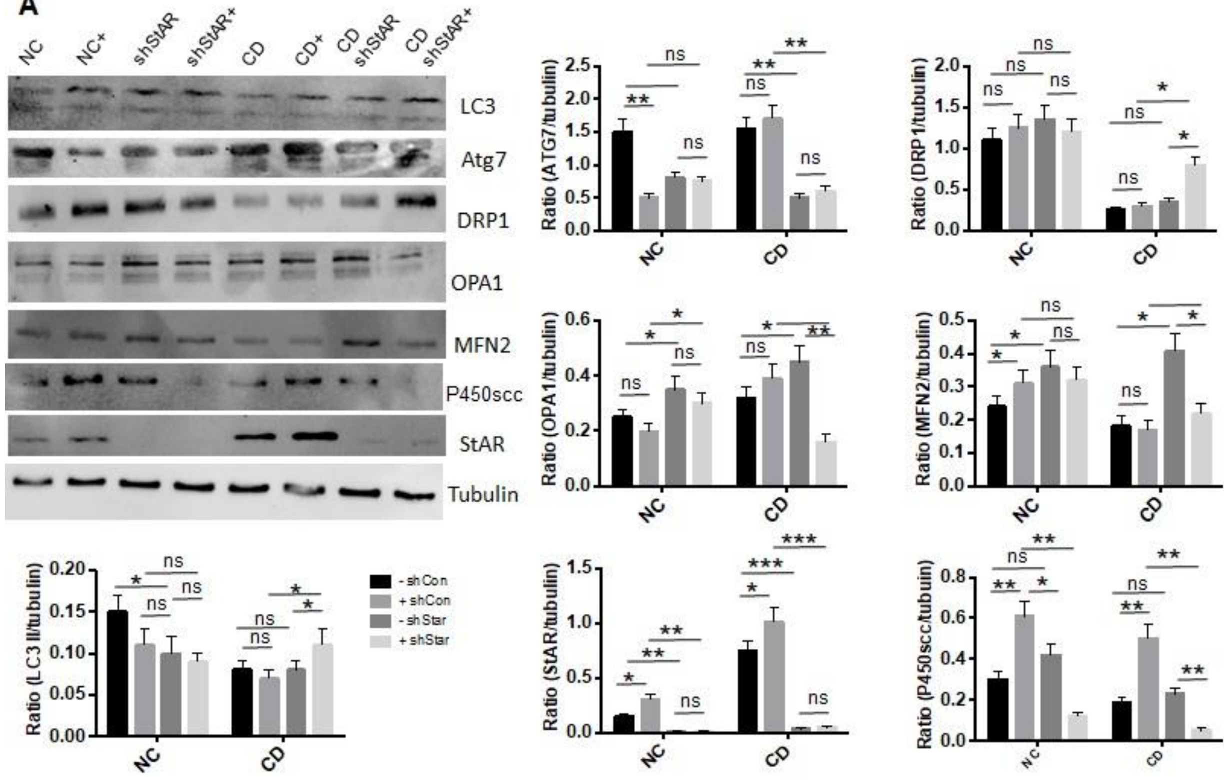


Figure 5.9. Ultrastructural analysis of MA-10 and Y-1 cells after augmenting intracellular cholesterol pool.

Upper panel: Representative transmission electron micrographs (magnification: 50000x) showing the effect of shRNA-mediated StAR knockdown in MA-10 and Y-1 cells on intracellular lipid droplets and mitochondria. Scale bars = 500 nm. Sc – scramble control, sh – shStAR, M – mitochondria; LD – lipid droplets; Ly – lysosome. Asterisk indicates sign of autophagy/mitophagy. Lower panel: Histograms showing quantification of LDs and mitochondrial number and size. Data are presented as mean ± SEM.

Moreover, we also analyzed cells lysates (from StAR knockdown MA-10 and Y-1 cells) for markers of autophagy. StAR knockdown was 90% as shown in blots and was found to differently affect LC3-II levels in cellsunder NC and CD experimental conditions. For example, in MA-10 cells, StAR knockdown was found to increase LC3-II level under hormonal stimulation, which was not observed in cells underNC condition (Fig. 5.10). Whereas an opposite effect was observed in Y-1 cells under similar condition. (Fig. 5.10). Moreover, ATG7 levels also displayed differences between scCon and shStAR groups under NC and CD condition in MA-10 and Y-1 cells (Fig. 5.10). Moreover, no difference was found in the expression level of mitochondrial dynamics markers (i.e., OPA1, DRP1 and MFN2) between different experimental groups in Y-1 cells, particularly without steroidogenic stimulation (Fig. 5.10). Interestingly, in MA-10 cells, StAR knockdown showed significant decrease in P450 levels even under hormonal stimulation, which was not observed in Y-1 cells (Fig. 5.10). This data, along with data obtained from the CD group, indicate that the roleof cholesterol in autophagy and mitochondrial dynamics is context-specific, includingwith/without hormonal stimulation and availability of steroidogenic cholesterol pool, which vary in different steroidogenic cell types.


A
NC
NC+
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shStAR+
CD
CD+
CD shStAR
CD shStAR+
LC3
Atg7
DRP1
OPA1
MFN2
P450scc
StAR
Tubulin
Ratio (ATG7/tubulin)
Ratio (DRP1/tubulin)
Ratio (OPA1/tubulin)
Ratio (MFN2/tubulin)
Ratio (LC3 II/tubulin)
Ratio (StAR/tubulin)
Ratio (P450scc/tubulin)
- shCon
+ shCon
- shStar
+ shStar

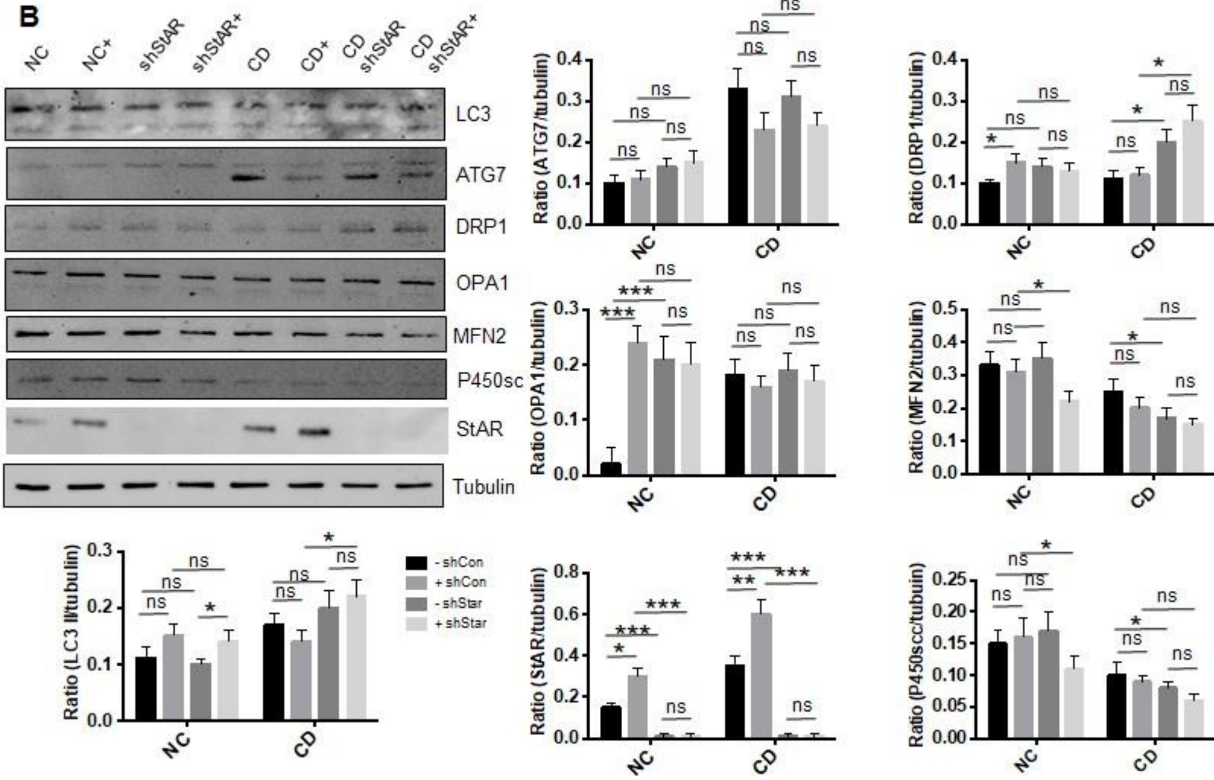
B
NC
NC+
shStAR
shStAR+
CD
CD+
CD shStAR
CD shStAR+
LC3
ATG7
DRP1
OPA1
MFN2
P450sc
StAR
Tubulin
Ratio (ATG7/tubulin)
Ratio (DRP1/tubulin)
Ratio (OPA1/tubulin)
Ratio (MFN2/tubulin)
Ratio (LC3 II/tubulin)
Ratio (StAR/tubulin)
Ratio (P450scc/tubuin)
- shCon
+ shCon
- shStar
+ shStar

Figure 5.10.

Immunoblots showing changes in protein levels of autophagy, mitochondrial dynamics and steroidogenic markers in response to StAR knockdown-mediated increased accumulation of lipid droplets/intracellular cholesterol pool in MA-10 **(A)** and Y-1 cells **(B)**. Quantification of protein band densities are shown with histograms (n = 3). Legend in LC3 II histograms is applicable to all histograms. NC – cells cultured under normal condition; CD – cell cultured under cholesterol depleted condition; ± indicates hormonal stimulation; shStAR – StARspecific shRNA; sc – scrambled control.

5.3.5 CD-induced changes sustain basal steroidogenesis

Finally, we examined the functional consequences of CD-induced changes on steroidogenesis in different steroidogenic cell types (i.e., the MA-10, Y-1 and BeWo cells) in the presence and absence of respective hormone stimulation at 6h, 12h and 24h. For this, we measured pregnenolone levels in all three cell lines for better comparison between different steroidogenic cell types rather than comparing their specific products. In MA-10 cells, CD led to a substantial increase in pregnenolone production when compared to the control group (NC) without steroidogenic stimulation at 6h (Fig. 5.11), which was maintained at 12h and subsequently decline at 24h (Fig. 5.11). Such changes were not observed in the NC group without steroidogenic stimulation, which remain similar during 6h–24h period (Fig. 5.11). In the presence of steroidogenic stimulation, although an increase in pregnenolone production was observed in the CD group, the pattern remains similar to the unstimulated CD group (Fig. 5.11). In the NC group, a consistent increase in steroid hormone production was found at all three time points (Fig. 5.11), which made it consistent with reports in existing literature [Light et al., 2015 and Turcu et al., 2015]. In Y-1 cells, CD increased steroid hormone production was sustained during 6h-24h, which was further increased in response to ACTH stimulation at 6h and 12h but not at 24h (Fig. 5.11). Moreover, a difference in the amplitude of pregnenolone production was observed under CD compared with the control groups (NC) in three different steroidogenic cell types, which were higher during 6h-12h and then decline at 24h (Fig. 5.11). In BeWo cells, CD in combination with hCG treatment led to a substantial increase in pregnenolone production at 6h and 12h but not 24h (Fig 5.11). In aggregate, our findings suggest a relationship between the intracellular cholesterol pool, the mitochondria, and steroidogenic capacity, which vary in different steroidogenic cell types due to their diverse physiological needs of different steroid hormones.

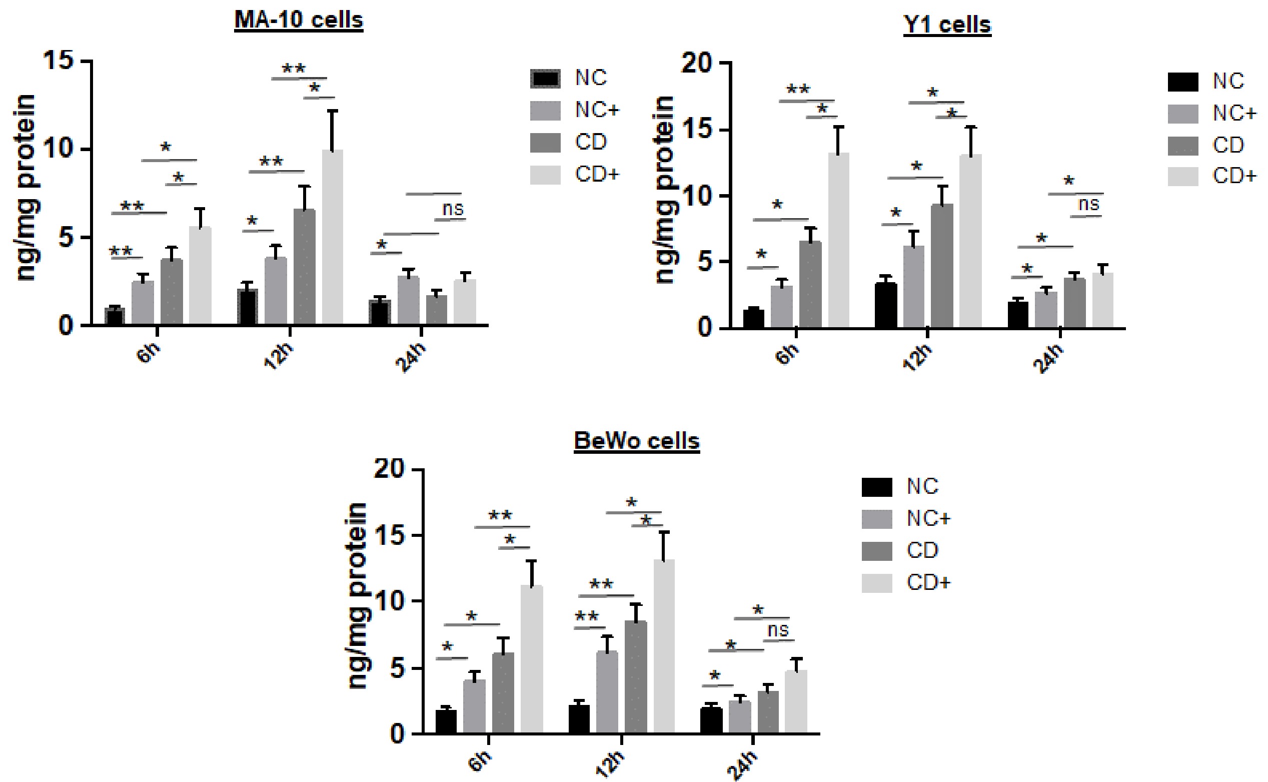

Figure 5.11. Cholesterol deprivation-induced changes in steroidogenic cells sustain basal steroidogenesis.

Histograms showing pregnenolone production from MA-10 cells, Y1 cells and BeWo cells in response to cholesterol deprivation with/without hormone stimulation. NC – normal culture condition, CD – cholesterol depleted culture condition. H – hormone treatment (hCG or ACTH, as applicable), ns – not significant, Data are presented as mean ± SEM (n = 3).

5.4 Discussion

This study reports that the intracellular cholesterol pool in steroid hormone-producing cells plays a role in the regulation of steroidogenic events and factors, and consequently, in steroid hormone production. Our findings are consistent with a previous report that decreasing cholesterol/phospholipids molar ratio by increasing phospholipid levels in the plasma membranes affect steroidogenesis in Y-1 adrenocortical cells [Iida et al., 1989]. Our overlapping finding of the effects of altering intracellular cholesterol pool on cell signaling intermediates involved in steroidogenesis and consequently on steroidogenic factors and on the markers of steroidogenic events, including autophagy and mitochondrial dynamics in steroidogenic MA-10, Y-1 and BeWo cells, but not in non-steroidogenic H9c2 cells, would imply that the observed effects are pertinent to steroidogenesis, especially basal steroidogenesis. Whereas differences observed in steroidogenic signaling and factors between MA-10, Y-1 and BeWo cells at a specific time point (i.e., 6h, 12h and 24h) are likely due to differences in the steroidogenic machinery, such as their cell-intrinsic structural differences (e.g., mitochondrial structure, number, and the varying steroidogenic cholesterol pool/lipid droplets) and steroidogenic capacity. Similarly, differences observed between different time points in a particular steroidogenic cell type are likely due to temporal and dynamic changes in steroidogenic factors and events (e.g., activation levels of cell signaling intermediates, changes in their cholesterol pool, as well as expression levels and functions of different proteins involved in cholesterol uptake and handling for steroidogenic need). In addition, we demonstrated the feasibility of selectively probing the role of cholesterol in steroidogenic cell type-specific functions using a simple experimental approach without affecting the basic cellular need of cholesterol (as reflected in cell viability assay), which is challenging to achieve using chemical (e.g., methyl-β-cyclodextrin-mediated) or genetic approaches (e.g., perturbing de novo biosynthesis).

An increase in p-PKA and/or p-ERK1/2 levels in different steroidogenic cell types in response to cholesterol deprivation (independent of trophic hormone stimulation) would imply that cholesterol deficiency or insufficiency, in some way, leads to the activation of upstream signaling involved in steroidogenesis, which may be a compensatory response to maintain basal steroidogenesis. Moreover, the modulation of cholesterol deprivation-induced changes in different steroidogenic cells by respective trophic hormones indicate that a relationship exists between the intracellular cholesterol pool and the hormone ligand-induced activation of the G-protein coupled

receptor (GPCR)-mediated steroidogenic signaling, which likely works in a context-dependent manner (e.g., the absence or presence of trophic hormones and the available steroidogenic cholesterol pool). Intriguingly, trophic hormone receptors are localized in the cholesterol-rich environment in the cell (i.e., the cell membranes) [Ulloa-Aguirre et al., 2018] whereas the steroidogenic enzymes are localized in a cholesterol-poor environment (i.e., the IMM and smooth ER) [Miller, 2008]. It is conceivable that this unique set-up in steroidogenic cells, including cholesterol transport to the mitochondria as a committed step, makes cholesterol a critical player in the regulation of steroidogenesis (more than a precursor substrate for steroid hormones) across steroidogenic cell types (Fig. 5.12), which will require further investigations to confirm. The physiological and pathophysiological implications of our findings are broad. For example, a similar mechanism might operate during the initiation of trophic hormone-independent adrenocortical and testicular steroidogenesis in the course of fetus development [Melau et al., 2019], as well as in steroid hormone-producing cancer cells [Mahata et al., 2020], including recently reported role of cholesterol synthesis in cancer metastasis [Han et al., 2022], which warrants further investigations.

Furthermore, as changes related to mitochondrial dynamics in different steroidogenic cell types (in this study) appeared in an overlapping manner alongside autophagy-related changes, it is possible that changes related to autophagy and mitochondrial dynamics are interlinked and may be a stepwise and integrated response to sustain steroidogenesis under varying cholesterol availability and steroidogenic needs (e.g., basal and hormone induced) (Fig. 5.12).

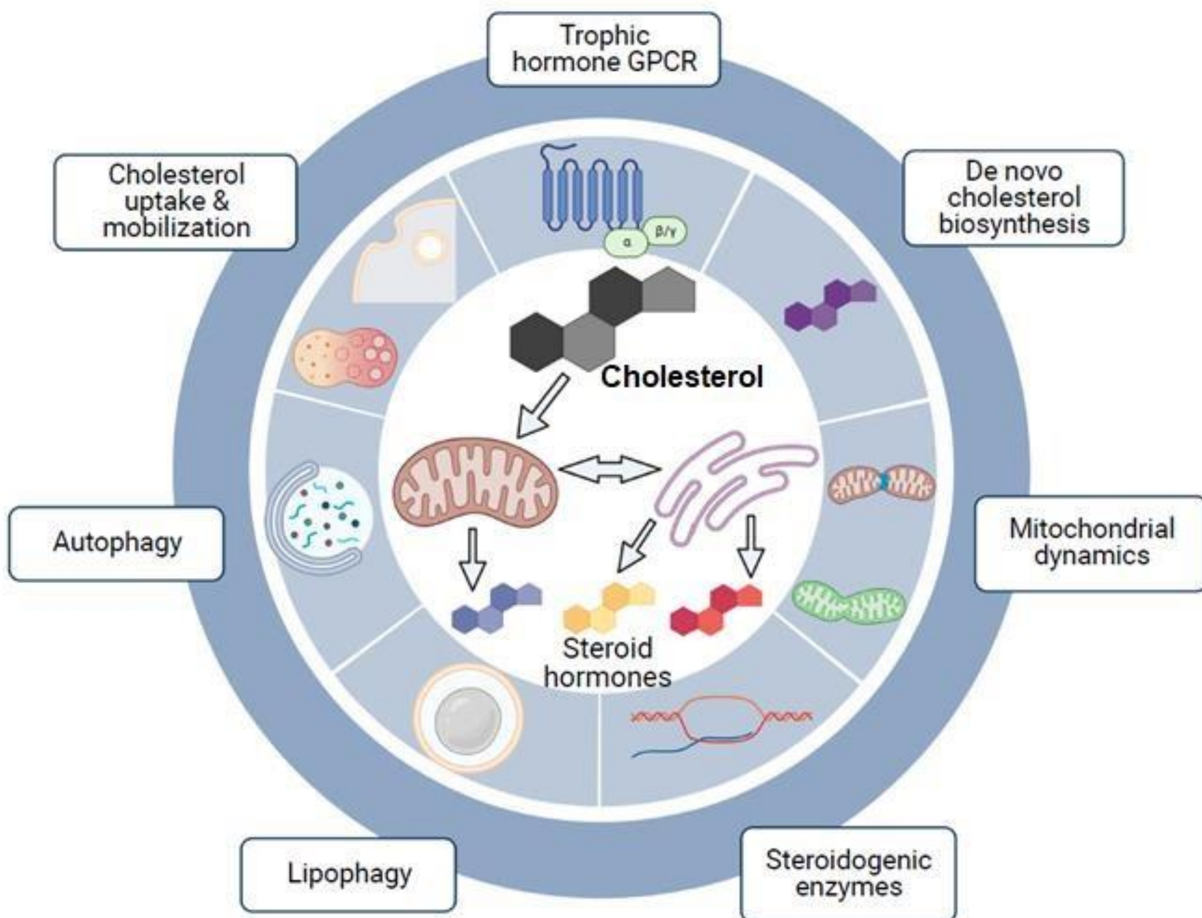

Figure 5.12. Cholesterol – more than a substrate for steroid hormones.
Our findings provide a proof of principle that cholesterol plays a role in the regulation of steroidogenic factors and events, which had not been demonstrated before. We propose that the unique set-up of cholesterol in steroidogenic cells, including a precursor substrate, heterogeneous distribution in organelle membranes with steroidogenic link (i.e., plasma membrane, mitochondrial membranes and smooth ER) and preexisting pool to support acute steroidogenesis makes it a unique and critical regulator of basal and trophic-hormone-induced steroidogenesis. Future studies warranted to better understand cholesterol-centric set-up of steroidogenesis.

The precursor cholesterol for steroidogenesis is known to come from at least three different sources, including mobilization of cholesterol from the plasma membranes / intracellular LDs, the uptake of circulating cholesterol esters (in the form of lipoproteins), and the *de novo* synthesis of cholesterol [Freeman et al., 1989 and Venugopal et al., 2021]. In addition, autophagy-mediated cholesterol trafficking has been implicated in steroid production [Texada et al., 2019]. Among different sources, plasma lipoproteins are the major source of cholesterol for steroidogenesis. For example, low-density lipoprotein accounts for about 80% of cholesterol delivered to the steroidogenic gland for steroidogenesis [Connelly et al., 2003]. Moreover, *de novo* synthesis of cholesterol from acetate do occurs in steroidogenic cells; however, its relative contribution to steroid hormones produced is less. However, it is conceivable that relative contribution by de novo cholesterol synthesis may substantially increase in the absence of lipoprotein uptake. A readily available pool of free cholesterol in the steroidogenic cells exist, which serve the basal and acute production of steroid hormones. In addition, steroidogenic stimulation increases hydrolysis of stored cholesterol esters to free cholesterol, increase uptake from plasma lipoproteins, and increased cholesterol biosynthesis within the gland [Tsujishita et al., 2000]. The acute response to a steroidogenic stimulation is mediated by the StAR protein, which facilitate cholesterol transport across the OMM. However, our understanding of the relative contributions of various sources of cholesterol to the different stages of steroidogenesis (i.e., basal, acute, and chronic) in major steroidogenic cells remains limited. It is likely that these processes work in a coordinated manner to maintain the physiological needs of different steroid hormones, which vary substantially under basal and stimulated conditions. The mobilization of cholesterol from LDs may play a major role in the acute response to trophic hormones, whereas the *de novo* synthesis of cholesterol may be primarily involved in basal and chronic steroidogenesis, whereas the cellular uptake of cholesterol in replenishing the depleted, readily available pool due to the acute response, and in the maintenance of a chronic response. By the same token, the instantaneous contribution of the *de novo* synthesis of cholesterol for acute steroid production in response to trophic hormones is expected to be minimal. Our findings provide a proof of principle that cholesterol is more than a substrate for steroid hormones and plays a role in the regulation of steroidogenic factors and events (Fig. 5.12). Future studies warranted to better understand the multifaceted relevance of cholesterol-centric set-up of steroidogenesis.

CHAPTER 6. CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

6.1 Conclusion

In addition to a well-known role of PHB1 in mitochondrial biology and lipid metabolism, my investigations identified PHB1 as a target gene for luteinizing hormone and revealed that PHB1 plays a regulatory role in Leydig cell steroidogenesis to maintain testosterone production and protects the hypothalamic-pituitary-testicular axis by preventing hormone insufficiency and excess. This appears to involve a coordination between the cell compartment- specific functions of PHB1, including membrane signaling and mitochondrial functions and predominantly involves Tyr114 residue in PHB1. This function is dysregulated in mPHB1 mice leading to increased testosterone production and consequently decreased gonadotropin levels, likely due to negative feedback inhibition at the hypothalamus and pituitary levels. Thus, signifying the importance of intracellular events in steroidogenic cells and their relationship with hypothalamic-pituitary axis to maintain normal steroid hormone production. Additionally, my findings identified interaction of PHBs (i.e., PHB1 and PHB2) with proteins involved in cholesterol transport across the OMM (i.e., StAR) and its subsequent utilization by the enzyme P450scc in the IMM. This finding alongwith data on direct interaction of PHB1 and cholesterol would imply a potential role of PHB1 (and its heterodimeric megacomplex with PHB2) in facilitating cholesterol transport acrossmitochondrial membranes for steroidogenesis. I anticipate that further investigations into the localization and function of a novel inner mitochondrial membrane protein PHB1 will significantly add to the understanding of an elusive step in steroidogenesis (i.e., cholesterol trafficking to the IMM).

Moreover, my finding of the interaction between StAR, PHB1 and mitochondrial proteases, as well as a dynamic relationship between StAR and PHB1 protein levels in response to steroidogenic stimulation are suggestive of a potential role of PHB1 in the acute regulation of StAR and consequently in protecting mitochondria and mitochondrial membrane from cholesterol toxicity (as mitochondrial membranes are poor in their cholesterol content and very sensitive changes in their cholesterol content). It is possible that PHB1 may play a role in the coordination of cell-neutral and cell type-specific function of mitochondria pertaining cholesterol trafficking and utilization. Thus, my findings have open new opportunities to get novel insights into cholesterol handling by steroidogenic cells to perform steroidogenic cell type-specific

function without compromising the fundamental need of cholesterol in membrane physiology, including organelle membranes.

In addition, exploration into the potential role of the intracellular cholesterol pool in steroidogenic events unraveled its role in basal steroidogenesis and potentially in modulating trophic hormone signaling in steroidogenic cells beyond a mere substrate for steroid hormones. Taken together, these findings open new research questions/directions and have created new opportunities to fill the knowledge gaps, answer some of the outstanding questions (as discussed in Appendix A/ Section 1.7) and advance our understanding of a fundamental aspect of steroidogenesis, which is highly conserved in many species.

6.2 Limitations

A major limitation of the first part of this study pertaining to the role of PHB1 in steroidogenesis in Chapter 4 is the use of only mouse models and murine cell lines. Further investigations are necessary to confirm the biomedical relevance of my findings in human physiology and pathophysiology. However, as the major finding of this study (cholesterol homeostasis and functional coupling of StAR and P450scc across the mitochondrial membranes) are similar in steroidogenic cells in both mice and humans, my findings are likely to have biomedical significance. In addition, a possibility exists that the *Fabp4* gene promoter that was used to developPHB-Tg and m-PHB-Tg mice may drive PHB1 expression in other cell or tissue type, such as testicular macrophages (which are present in the interstitial space along with Leydig cells) and hypothalamus, which may contribute to testicular phenotype observed in this study. However, a series of in vitro experiments taken in this study using PHB1 manipulated MA-10 cells (as well as absence of such alteration in the hypothalamic-pituitary gonadal axis in the female mice) stronglysuggest a role of PHB1 in Leydig cell biology.

In the second part of the thesis related to the role of the intracellular cholesterol pool in steroidogenic cells in Chapter 5, I have only used cell culture models. Again, further investigations are required to confirm the physiological and pathological significance of our findings of the role of intracellular cholesterol pool in the regulation of cell-intrinsic events under basal and trophic hormone-stimulated conditions. Further, to measure cholesterol, I have utilised Amplex^{TM} Red Cholesterol Assay, to showcase lipid droplets transmission electron microscopy was used, I could have also used Filipin and BODIPY immunofluorescence staining to further show the changes in cholesterol and lipid droplets in different treatment groups. Increased levels of LC3-II, an autophagy marker used in the study does not indicate autophagic flux at a particular time as it could either reflect increased autophagosome formation or decreased autophagolysosomal degradation. Similarly, other experimental approaches are needed to better define the interaction between PHB1 and cholesterol, autophagy, as well as with other proteins known to be involved in cholesterol trafficking, and processing/utilization across mitochondrial membranes.

6.3 Future directions

My findings of PHB1's role in Leydig cell steroidogenesis raises an obvious question — whether PHB1 has a similar role in other steroidogenic cells/tissues, such as adrenocortical cells, ovarian granulosa and theca cells, as well as in syncytial trophoblast cells in placenta. Moreover, since PHB1 has cell compartment-specific function, including membrane signaling and mitochondrial functions, it would be important to decipher the relative contribution of cell compartment-specificattribute of PHB1 in steroidogenesis. In addition, it would be interesting to know whether smaller testis size (despite increased testosterone levels) that was observed in mPHB1 expressing male mice is a consequence of lower gonadotropin levels or whether PHB1 has a developmental role.

Another area where PHB-1 could play an important role is the acute regulation of StAR. It has been previously shown that PHB-1 and its heterodimeric partner PHB-2 interact with various mitochondrial proteases in other model organisms. Moreover, similar proteases have been identified in relation to acute regulation of StAR. Thus, a possibility exists that PHB1 may play a role in the acute regulation of StAR, either alone or in collaboration with PHB2. It would be interesting to understand the role of mitochondria, mitochondrial proteins such as prohibitins and proteases in the regulation of StAR turnover. Further, StAR knockdown seems to be making global changes in the cellular machinery, performing RNA sequencing would be a good approach to analyze global gene-expression changes.

Leydig cell-specific knockout mouse model, including conditional knockout models of PHB1 and in combination knock-in mouse model and expression of transgenic mice under the Leydig cell specific promoter INSL3 are expected to shed new light on the physiological relevance of PHB1 in steroidogenic cells in different steroidogenic tissues.

Recent advances in the field of autophagy introduced mitophagy and lipophagy as key players in the regulation of intracellular cholesterol trafficking and homeostasis in steroidogenesis. However, it is unclear how these homeostatic processes regulate steroidogenesis under basal and hormone-stimulated conditions in different steroidogenic tissues with different and wide-ranging physiological demand. Using ATG7 knockdown steroidogenic cells can give a better understanding of how autophagy plays a role in regulating cholesterol uptake and trafficking. Further, comparing the LC3-II levels in the presence or absence of lysosomal protease inhibitor

(Bafilomycin A1) will indicate autophagic flux in real time. A better understanding of the mechanisms involved in governing such processes will provide new insight in the field and could be beneficial in developing novel therapeutic avenues.

Furthermore, differential effects of the depletion of intracellular cholesterol pool on steroidogenic events and factors under basal and trophic hormone-stimulated conditions in different steroidogenic cell types have open new research directions in the field of steroidogenesis, which necessitate further investigations.

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