Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Magee-Womens Research Institute and Foundation

2. **Reporting Period (start and end date of grant award period):** 1/1/13-12/31/13

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Cheryl A Richards, MBA

4. **Grant Contact Person's Telephone Number:** 412-641-8932

5. **Grant SAP Number:** 41000622212

6. **Project Number and Title of Research Project:** Project 4 - Oxidative stress, hypoxia and placental cholesterol metabolism

7. **Start and End Date of Research Project:** 1/1/13-12/31/13

8. **Name of Principal Investigator for the Research Project:** Jacob Larkin, MD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 84,788.60

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Sun, Huijie	Research Technician	45%	$20,525
Witt, William	Research Technician	100%	$13,527

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Larkin, Jacob	Principal Investigator	5%
Sears, Sarah	Medical Student	5%
Powers, Robert	Collaborator	1%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes____X_____ No_____

If yes, please indicate the source and amount of other funds:

AAOGF/SMFM Research Scholarship
$100,000/year (direct) (Grant ended 6/30/13)

NIH K12HD063087
$125,000/year (Grant began 7/1/13)

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____X____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Placental injury and cholesterol metabolism	☒NIH (K12) ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	3/2013	$384,000	$384,000
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____X____ No_____

If yes, please describe your plans:
I will resubmit a K99/R00 application to the NIH in March, 2014.

12. Future of Research Project. What are the future plans for this research project?

Work commenced for this project will continue, with applications in vitro and in mice. We will begin detailed analysis of mechanisms regulating the activity of the Liver X Receptor (LXR) and Sterol-Response Element Binding Protein 2 (SREBP2) in human trophoblasts, and the effect of these regulatory mechanisms on placental function.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____X____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown			1	
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White			1	
Black				
Asian				
Other				
Unknown				
Total			**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____X___ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

Funding is a critical determinant of progress in research. With funds provided by the health research project, I was able to hire a lab technician. His presence contributed to the 'critical mass' of people engaged in research. Furthermore, we developed novel techniques of tracing cholesterol trafficking in human trophoblasts. This line of investigation had never been conducted at our institution prior to the past year.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No_____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.
List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons

why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Oxidative Stress, Hypoxia and Placental Cholesterol Metabolism – While oxidative stress and hypoxia are known to cause placental injury and to disrupt cholesterol homeostasis, their effect on placental cholesterol metabolism is not known. We posit that disruption of placental cholesterol homeostasis adversely affects placental function and fetal growth. Our focused interrogation of placental cholesterol metabolism and its effect on fetal growth will elucidate central pathways in placental metabolic function, and highlight therapeutic targets for the amelioration of placental injury.

Project Overview

Fetal growth and development are dependent on the placenta, which mediates nutrient and gas exchange, removal of waste products, immunological protection of the allogeneic fetus, and pregnancy-specific endocrine signaling. Placental injury and subsequent dysfunction lead to fetal growth restriction (FGR), and confer risk for perinatal death, neonatal morbidity, neurodevelopmental disorders, and adult disease. Despite the associated risks, a large knowledge gap persists regarding the pathobiology of placental dysfunction and FGR. The only

therapeutic option for women with pregnancies affected by FGR is delivery, often compounding underlying pathology with the sequelae of iatrogenic prematurity.

Cholesterol is essential for the integrity of cellular membranes, and plays a pivotal role in multifold synthetic and signaling pathways. Cholesterol homeostasis can be disrupted by oxidative stress or hypoxia, and limited prenatal cholesterol availability is associated with impaired fetal growth and developmental abnormalities. Whereas hypoperfusion and the resultant hypoxia are common causes of placental injury, the impact of oxidative stress and hypoxic injury on placental cholesterol metabolism, and the subsequent effect on placental function and fetal growth, remain unknown.

As a part of our long-range goal of elucidating pathways that define trophoblast dysfunction, we seek to test the hypothesis that hypoxia and oxidative stress disrupt placental cholesterol trafficking and metabolism, leading to impaired trophoblast function and restriction of fetal growth. We will test this hypothesis using the following Aims:

Aim 1 – Probe the regulation of placental cholesterol homeostasis, and determine the effect of hypoxia and/or oxidative stress on placental cholesterol processing and oxidation

Aim 2 - Probe the effect of hypoxia and oxidative stress on expression of transcriptional regulators and effectors of cholesterol metabolism.

Expected Research Outcomes and Benefits

Intracellular concentrations of cholesterol and its metabolites regulate ligand-binding and activation of multiple sterol-responsive transcription factors. Thus, the potential sequelae of aberrant regulation of sterol metabolism are far-reaching. We posit that disruption of placental cholesterol homeostasis could adversely impact fetal growth via the following mechanisms:

1. Reduced maternal to fetal cholesterol transport.
2. Sterol-induced cytotoxicity causing placental cell death and compromise of function.

Cholesterol is a vital determinant of multiple diverse biological pathways, and a critical substrate for growth. The importance of maternal-fetal cholesterol transport for normal fetal development has been definitively established, highlighting the need for novel insights into cholesterol trafficking between mother, placenta and fetus. Nonetheless, the regulation of placental cholesterol metabolism and its impact on placental function and fetal growth remain largely unknown. In the proposed studies, we will interrogate the role of placental cholesterol homeostasis in placental biology with a focus on the mechanisms of action detailed above. We will complement experiments in primary human trophoblasts with analysis of human specimens and *in vivo* studies of pregnancy in mice. Our work will decipher critical components of prenatal metabolic regulation and is likely to illuminate the pathophysiology of fetal growth restriction that stems from placental metabolic injury in an unprecedented manner.

Summary of Research Completed

We have met our goals and accomplished our stated aims. Through the work supported, we have identified relevant cholesterol acceptors for efflux of cholesterol from human trophoblasts, uncovered novel effects of hypoxic injury on maternal-fetal cholesterol homeostasis, and gained significant new insight into the impact of oxidized cholesterol products on trophoblast toxicity, differentiation, progesterone synthesis, and gene expression. Work performed delineating pathways of cholesterol efflux was supported specifically by Pennsylvania Department of Health (PA DOH) Formula Health Research Funds (2012). Studies of gene expression and progesterone synthesis in human trophoblasts were supported by Health Research Formula Funds, as well as the AAOGF/SMFM Research Scholarship and the NIH K12HD063087 grant. Our findings underscore the potential importance of cholesterol metabolism and oxidation on basic trophoblast function and point to the liver X receptor (LXR) as a potential therapeutic target that can be ligand-activated with gene target and functional specificity.

Milestones for 7/1/2013-12/31/2013

Milestones set with the initial application for funds are listed below in italics, followed by the comments addressing the status of completion.

<u>Aim 1</u>

By 9/30/2013:

- *Complete analysis of cholesterol uptake, synthesis, esterification and efflux assays in PHT cells*
  - Efflux analysis has been completed. We have designed protocols for other components of cholesterol trafficking, begun data collection, and will complete analysis in the coming months.
- *Complete breeding, sample collection and sex determination of mouse embryos and placentas exposed to diquat, with appropriate controls.*
  - We have determined that exposure of pregnant dams to 10mg/kg intraperitoneal diquat from E12.5-18.5 causes a 20% reduction in embryo weight.

By 12/31/2013:

- *Complete comparison of fetal serum cholesterol and triglycerides, feto-placental growth, and placental immunostaining in mice.*
  - All breeding and sample collection has been completed on schedule. Assays and data analysis are ongoing currently.

<u>Aim 2</u>

By 9/30/13:

- *Complete gene expression and LXR/SREBP luciferase reporter experiments in PHT cells.*
  - We have delineated gene expression changes elicited by oxysterols and synthetic LXR ligands, as shown in Fig. 11, and correlated these alterations in gene expression with specific functional effects in human trophoblasts (Fig. 1-10). Furthermore, we have cloned the promoter sequences for two key LXR targets, fatty acid synthase (FAS) and ATP-Binding Cassette Transporter A1 (ABCA1) and designed luciferase reporters driven by these promoters. We have successfully transfected cells from the placentally-derived JEG-3 cell line with these reporter constructs. However, we have faced difficulty

achieving efficient transfection into primary human trophoblasts (PHTs). We plan to utilize lentiviral vector to improve expression of these reporters in PHTs.

- *Complete protein expression analysis in human placental biopsies.*
  - We have performed immunohistochemistry and in-situ hybridization to localize LXR expression in human placental biopsies.

By 12/31/2013:

- *Complete gene expression comparisons in mouse placentas.*
  - We have identified a group of apolipoproteins that are targets of LXR and differentially regulated in response to hypoxia in the placental labyrinth. These findings were published, and the Pennsylvania Department of Health was acknowledged as a funding source (Larkin, et al., Endocrinology, March 2014, 155(3):1099-1106).

Aim 1: – Probe the regulation of placental cholesterol homeostasis, and determine the effect of hypoxia and/or oxidative stress on placental cholesterol processing and oxidation.

Exposure of primary human trophoblasts (PHT) to 25-hydroxycholesterol (25OHC), a product of cholesterol oxidation, inhibited trophoblast differentiation and syncytialization after 48 and 72 hours in culture, as indicated by levels of human chorionic gonadotropin (hCG) secretion (Fig. 1). In contrast to prior publications showing a reduction in 25OHC-mediated cytotoxicity resulting from activation of the LXR with a synthetic ligand (Aye, et al, *Biochimica et Biophysica Acta*, 2010), we found that exposure of PHT cells to 10μM of the LXR ligand T0901317 exacerbated the reduction in hCG production at all measured doses of 25OHC. Similarly, increasing doses of 25OHC led to increasing levels of cell death, quantified through measurement of LDH released into the cell medium, which was further exacerbated by exposure to 10μM T0901317 (Fig. 2).

Despite the inhibition of differentiation and increased cytotoxicity, lower doses of 25OHC led to increases in the levels of measured progesterone concentration in cell medium (Fig. 3). However, as shown in Fig. 3 at higher doses of 25OHC, progesterone synthesis in PHT cells was impaired. Addition of 10μM T0901317 to cell medium altered the relationship between 25OHC level and progesterone synthesis, causing a reduction in the dose of 25OHC at which progesterone synthesis declined (measured at 48 and 72 hours in culture). We performed additional experiments to determine the effect of hypoxic exposure on progesterone synthesis. Cells were initially plated at standard oxygen tension (21% O_2) in standard medium with 10% fetal bovine serum. Twenty-four hours after plating, cells either remained at standard O_2 tension, or were transferred to an 8% or <1% O_2 environment. All medium was replaced with serum-free medium at 24 hours after plating. As shown in Figure 4, environmental O_2 levels significantly impact progesterone secretion in trophoblasts, with declining secretion at lower O_2 levels. Cells were treated with increasing doses of free cholesterol, low-density lipoprotein (LDL), or 25OHC (Fig. 5). Consistent with the findings shown in Figure 4, progesterone secretion was dramatically reduced in hypoxia. Furthermore, when exposed to O_2 tension below 1%, PHT cells demonstrated increased progesterone secretion with increasing substrate availability ($p<0.05$, test for trend for all substrates), while at 8% O_2, substrate availability had a variable effect on progesterone secretion, with no significant trend detected.

The discrepant effect of 25OHC and LXR activation on trophoblast toxicity and differentiation on the one hand, and progesterone synthesis on the other, suggests that placental steroid hormone synthesis is not simply a function of trophoblast viability and differentiation and is determined by levels of oxidized cholesterol and LXR activation.

In addition to the findings above, as outlined in our initial proposal and with the support of Pennsylvania DOH funds, we have developed a technique in PHT cells that permits measurement of cholesterol efflux, a function that is potentially vital to sustaining fetal growth and development. PHT cells are plated in 24-well plates at a density of 150,000 cells/well. For the first 24 hours in culture, cells are incubated with medium containing 10% bovine growth serum and tritiated cholesterol (0.2 μCi/mL). After 24 hours of incubation, the medium is removed and the cells washed twice with PBS and incubated with Dulbecco's Modified Eagle Medium (DMEM) containing no serum and 1mg/mL fatty acid-free albumin (FAFA) for a 2-hour equilibration period. After equilibration, the cells are then incubated with efflux media containing DMEM with no serum, 1mg/mL FAFA, and varying concentrations of the cholesterol acceptors ApolipoproteinA1 (ApoA1) and/or high-density lipoprotein (HDL). If cells remain in culture for more than 24 hours beyond the completion of the equilibration period, identical fresh media is added 24 hours past completion of equilibration (48 hours after plating). For quantification of cholesterol efflux, medium is collected and centrifuged briefly to remove cell debris. Cultured cells are collected separately and lysed in PBS with 1% Triton X-100. The amount of tritiated cholesterol in the medium and cell lysate is quantified by liquid scintillation counting and the percent efflux is calculated as counts per minute in [cell medium/(cell medium + cell lysate)]. Parallel experiments were performed in the JEG-3 human placental choriocarcinoma cell line.

In early experiments supported by the Pennsylvania DOH (FY2012), we measured the effect of incubation with different cholesterol acceptors, altered levels of oxygen tension, and increasing concentrations of free cholesterol, 25OHC, and T0901317 on cholesterol efflux. As illustrated in Figure 6, in both PHT cells and JEG-3, addition of ApoA1 to the efflux media does not lead to any increase in cholesterol efflux after 48 hours in culture (24 hours past loading with tritiated cholesterol). In contrast, the addition of HDL led to a dramatic increase in cholesterol efflux. These findings suggest that lipid-poor ApoA1 does not serve as a cholesterol acceptor for efflux in human trophoblasts. We also measured the effect of increasing doses of T0901317 on cholesterol efflux in PHT cells. These experiments were performed in hypoxia (0% O_2), physiologic oxygen level (8% O_2), and standard culture conditions (21% O_2). As shown in Figure 7, in the absence of T0901317, increasing oxygen concentrations tend to promote cholesterol efflux.

In addition to *in vitro* experiments, as outlined in our initial proposal, we have determined the effect of hypoxia on maternal:fetal cholesterol transfer *in vivo*. We performed timed breeding of C57BL/6J males and females overnight, with separation the next morning, designated as embryonic day 0.5 (E0.5). Pregnancy is assumed based on a weight gain of 10% or greater at E10.5. Pregnant dams have *ad libitum* access to standard chow. To mimic hypoxic injury stemming from placental dysfunction, pregnant dams are placed in a 12% O_2 chamber at E12.5, while control mice remain in ambient air. Dams were sacrificed at E18.5, and serum was

collected from dams and embryos. Exposure to hypoxia caused a trend toward increased serum cholesterol in pregnant dams (30.0±3.37 (CTL) vs. 38.4±3.63 (hypoxia), p=0.103) with an opposite trend in embryonic serum cholesterol (35.2±1.34 (CTL) vs. 32.1±1.46 (hypoxia), p=0.129). These trends led to a significant reduction in fetal:maternal serum cholesterol ratio resulting from hypoxia (1.27±0.061 (CTL) vs. 0.947±0.076 (hypoxia), p=0.0012), suggesting a reduction in maternal:fetal cholesterol transfer resulting from hypoxic injury. The detected effect on the ratio of maternal:fetal serum cholesterol level was associated with altered expression of several lipid-transporting apolipoproteins in the placental labyrinth (Larkin, et al., *Endocrinology* 2014; en20131425. [Epub ahead of print].

Aim 2: Probe the effect of hypoxia and oxidative stress on expression of transcriptional regulators and effectors of cholesterol metabolism.

Milestones set for Aim 2 with the initial application for funds are listed below in *italics followed by the* comments addressing the status of completion in black.

Aim 2
By 9/30/13:

- *Complete gene expression and LXR/SREBP luciferase reporter experiments in PHT cells.*
  - We have delineated gene expression changes elicited by oxysterols and synthetic LXR ligands, as shown in Fig. 11, and correlated these alterations in gene expression with specific functional effects in human trophoblasts (Fig. 1-10). Furthermore, we have cloned the promoter sequences for two key LXR targets, fatty acid synthase (FAS) and ATP-Binding Cassette Transporter A1 (ABCA1) and designed luciferase reporters driven by these promoters. We have successfully transfected cells from the placentally-derived JEG-3 cell line with these reporter constructs. However, we have faced difficulty achieving efficient transfection into primary human trophoblasts (PHTs). We plan to utilize lentiviral vector to improve expression of these reporters in PHTs.
- *Complete protein expression analysis in human placental biopsies.*
  - We have performed immunohistochemistry and in-situ hybridization to localize LXR expression in human placental biopsies.

By 12/31/2013:

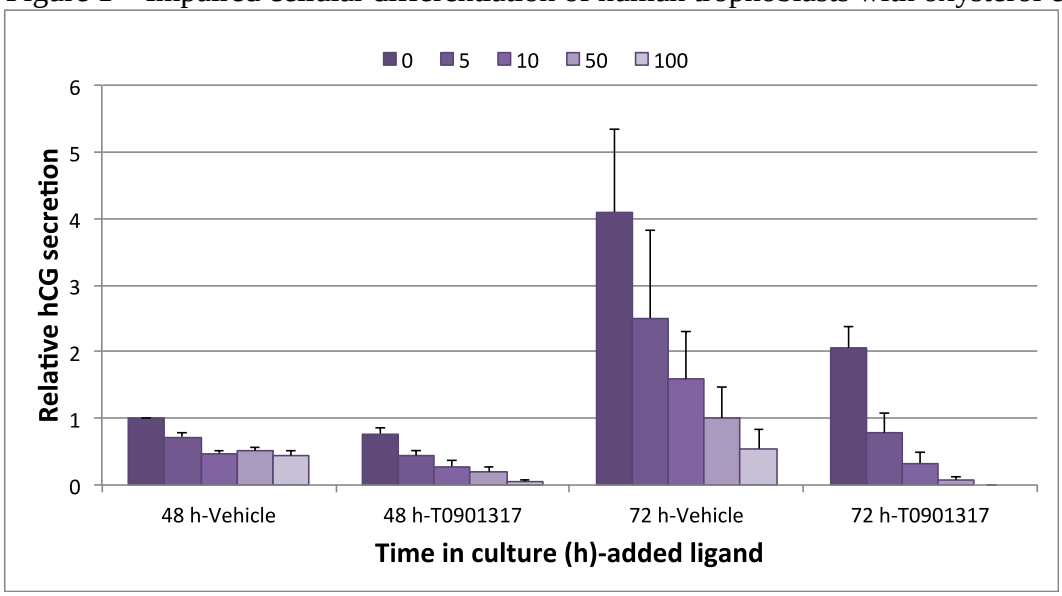
- *Complete gene expression comparisons in mouse placentas.*
  - We have identified a group of apolipoproteins that are targets of LXR and differentially regulated in response to hypoxia in the placental labyrinth. These findings were published and the Pennsylvania Department of Health was acknowledged as a funding source (Larkin, et al., Endocrinology, March 2014, 155(3):1099-1106).

As demonstrated in Figure 8, both 25OHC and the LXR-specific ligand T0901317 induce cell toxicity in a dose-responsive fashion. Similarly, both T0901317 and 25OHC inhibit trophoblast differentiation as determined by hCG secretion (please see manuscript 'The influence of ligand-activated LXR on primary human trophoblasts' listed in Item #20 for results and description of materials and methods). However, at 21% O_2, T0901317 elicits a dose-dependent reduction in progesterone secretion, while 25OHC has a variable effect on progesterone secretion at differing doses, with no discernable monotonic trend (Fig. 9). We also observed a discrepant effect of the

two LXR ligands on lipid droplet formation. T0901317 (10 μM) provoked robust lipid droplet formation comparable to that seen with hypoxic exposure (FiO_2<1%), while 10 μg/mL 25OHC had no detectable effect on lipid droplet formation (Fig. 10). To identify changes in gene expression that underlie the discrepant cellular effects of LXR ligands in human trophoblasts, we compared changes in gene expression elicited by these ligands. As shown in Figure 11, while T0901317 had no effect on expression of the Sterol-Response Element Binding Protein 2 (SREBP2) target LDL receptor (LDLR), exposure of PHT cells to T0901317 led to increased expression of ABCA1, SREBP1c, ACC1, and FAS. Similar to the effect of T0901317, exposure to 10 μg/mL of 25OHC did not significantly impact expression of SREBP2 or LXRβ and led to an insignificant increase in expression of LXRα. However, unlike T0901317, 25OHC selectively upregulated specific LXR targets, causing increased expression of the cholesterol efflux mediator ABCA1, with no effect on the expression of the lipogenic genes SREBP1c, ACC1, or FAS. Additionally, 25OHC inhibited expression of LDLR.

These findings indicate that not only does LXR activation influence specific trophoblast functions, but that it does so in a ligand-specific manner. The discrepancy in the functional and transcriptional effect of the two studied LXR ligands suggests that LXR is a potential therapeutic that can be targeted with selective ligands to activate specific targets and cell functions.

[1]Figure 1 – Impaired cellular differentiation of human trophoblasts with oxysterol exposure



Primary human trophoblasts (n=3) were treated with the indicated dose of 25OHC and 10μM T0901317 or control vehicle 24 hours after plating. hCG levels were measured at 48 and 72 hours after plating.

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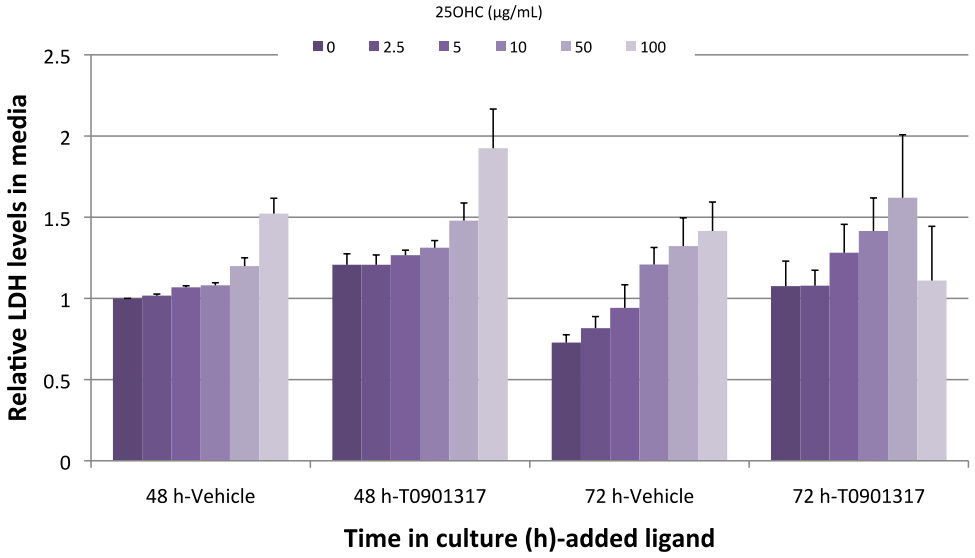
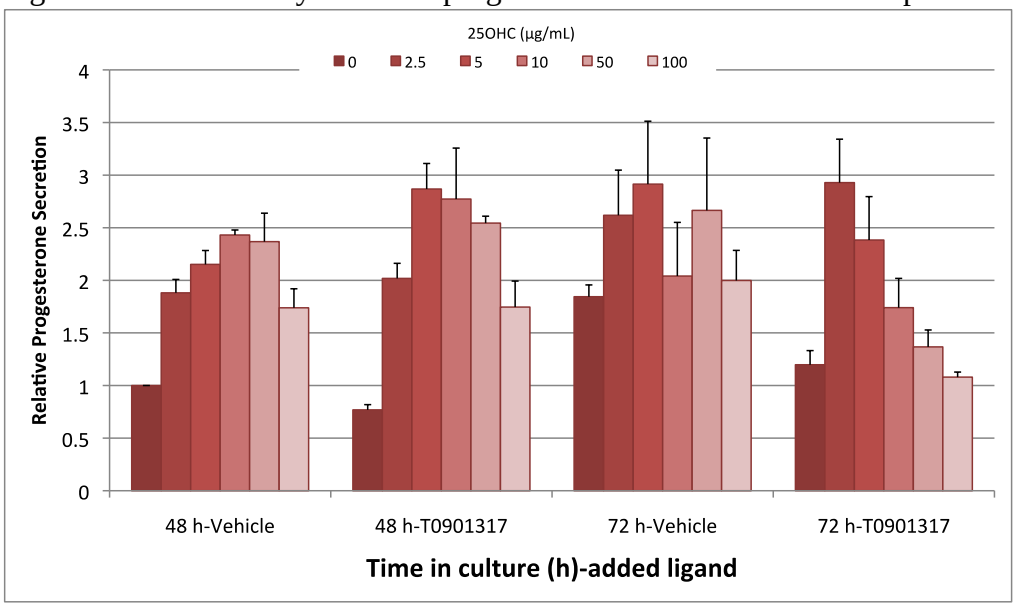
[1] For all figures, PHT=primary human trophoblast cells, 25OHC= 25-hydroxy cholesterol, hCG=human chorionic gonadotropin, LDH=lactate dehydrogenase, LDL=low-density lipoprotein, ApoA1=ApolipoproteinA1, HDL=high-density lipoprotein, SREBP=Sterol Response Element Binding Protein, LXR=Liver X Receptor, LDLR=LDL receptor, ABCA1=ATP Binding Cassette Transporter A1, ACC1=Acetyl-CoA carboxylase, FAS=Fatty Acid Synthase, RT-qPCR=reverse transcriptase quantitative PCR.

Figure 2 – Cytotoxicity caused by increasing levels of oxysterols in human trophoblasts



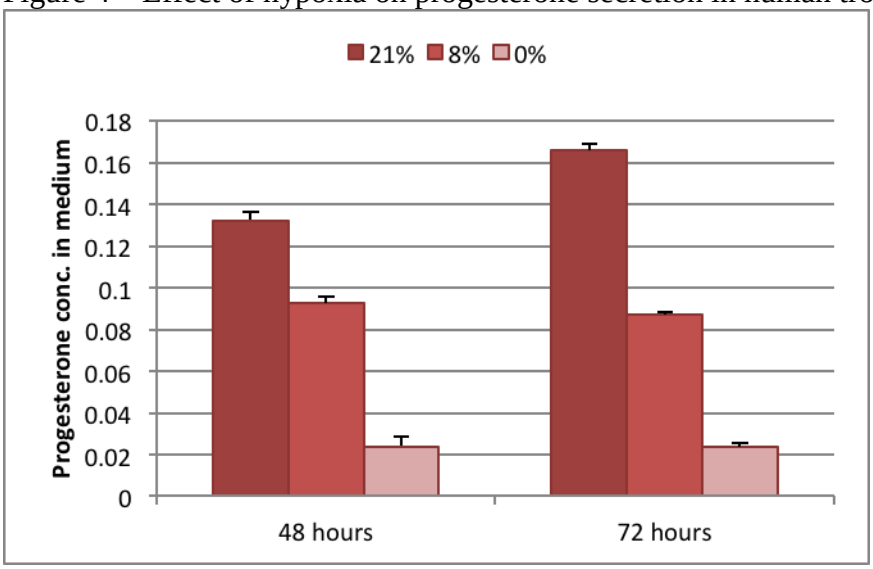
PHT cells (n=3) were treated with the indicated dose of 25OHC and 10µM T0901317 or control vehicle 24 hours after plating. LDH levels were measured at 48 and 72 hours after plating.

Figure 3 – Effect of oxysterols on progesterone secretion in human trophoblasts



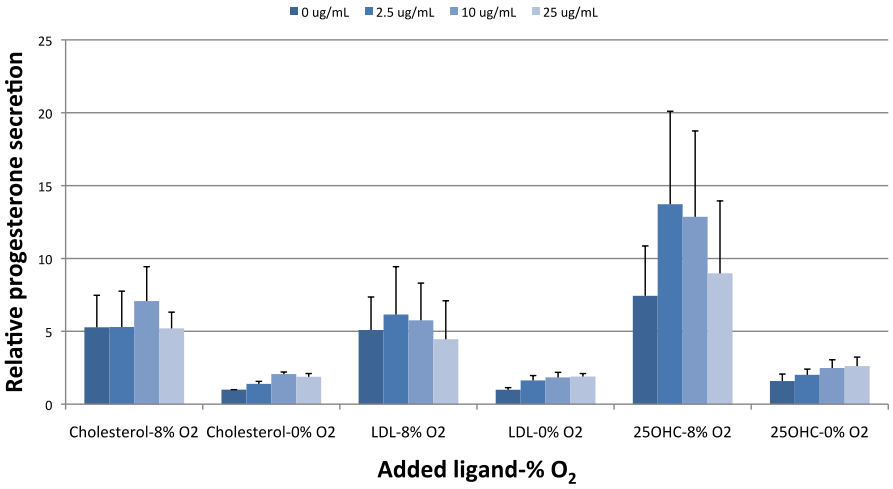
PHT cells (n=3) were treated with the indicated dose of 25OHC and 10µM T0901317 or control vehicle 24 hours after plating. Progesterone levels were measured at 48 and 72 hours after plating.

Figure 4 – Effect of hypoxia on progesterone secretion in human trophoblasts



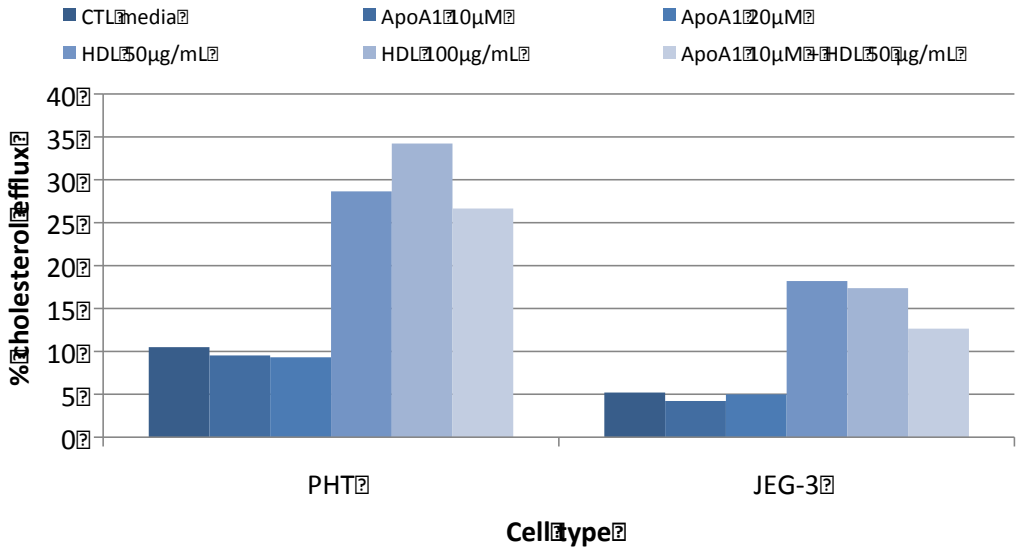
PHT cells (n=3) were cultured at indicated O_2 levels beginning 24 hours after plating. Progesterone levels were measured at 48 and 72 hours after plating.

Figure 5– Effect of ambient oxygen and substrate concentration on progesterone secretion



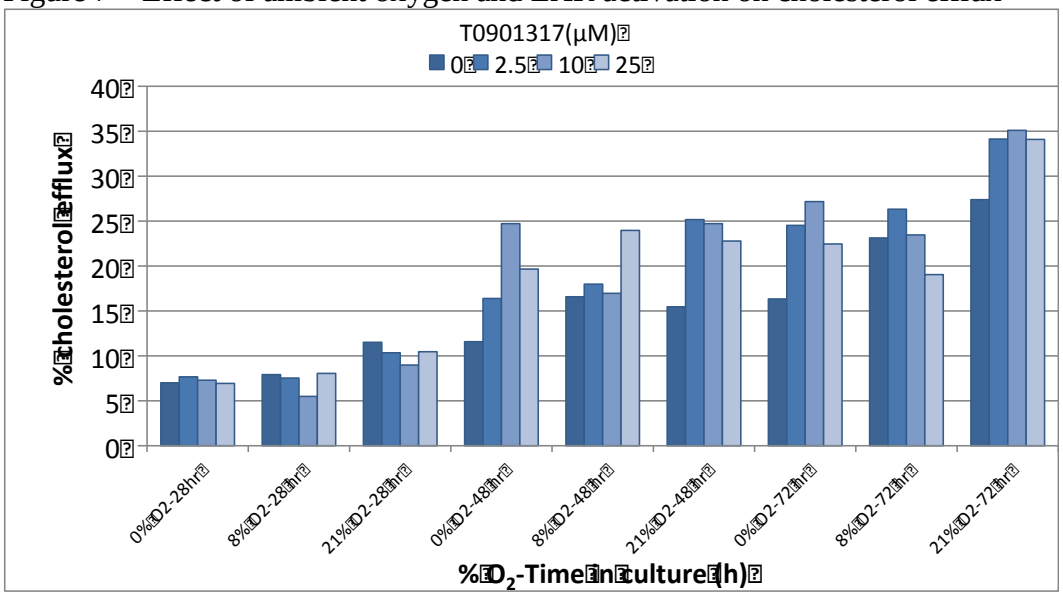
PHT cells were treated with the indicated dose of free cholesterol, LDL, or 25OHC and exposed to indicated O_2 tension. Progesterone concentration in cell media was measured 72 hours after plating.

Figure 6 – Effect of ApoA1 and HDL on cholesterol efflux in placental cells



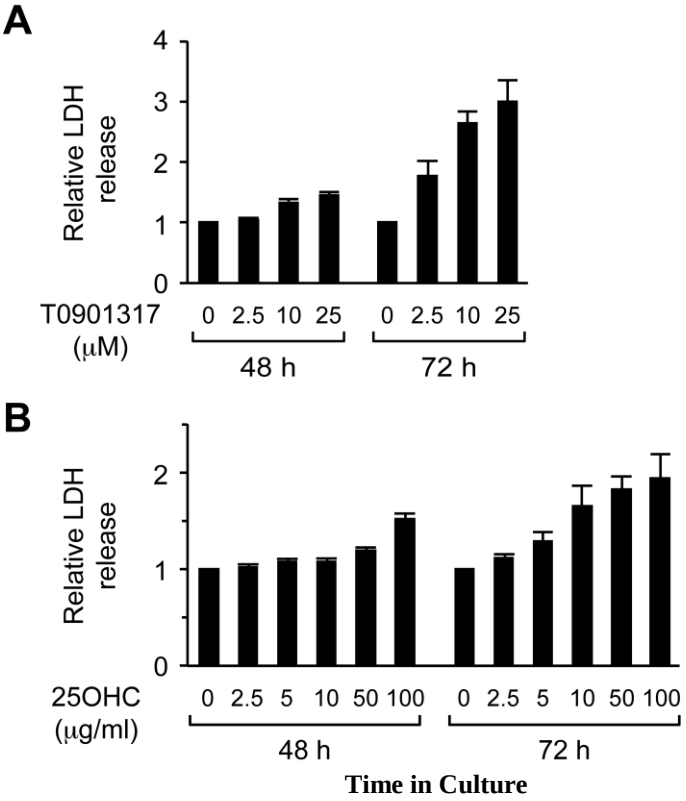
Efflux was measured 24 hours post-incubation with tritiated cholesterol.

Figure 7 – Effect of ambient oxygen and LXR activation on cholesterol efflux



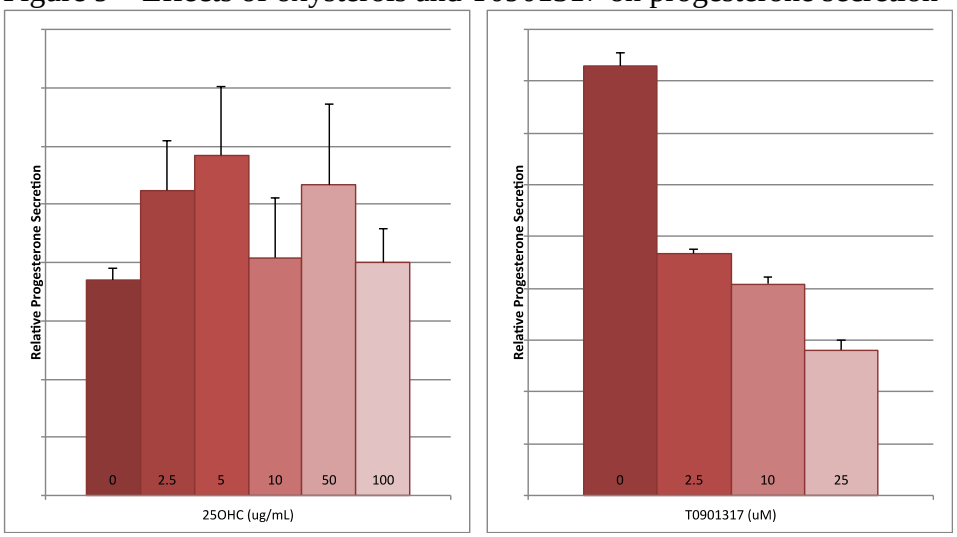
PHT cells were treated with the indicated dose of T0901317 and exposed to the indicated O_2 tension following incubation with tritiated cholesterol at 21% O_2 (24 hours after plating). Cholesterol efflux was measured at 28, 48, and 72 hours after plating.

Figure 8 – LXR ligands cause trophoblast toxicity



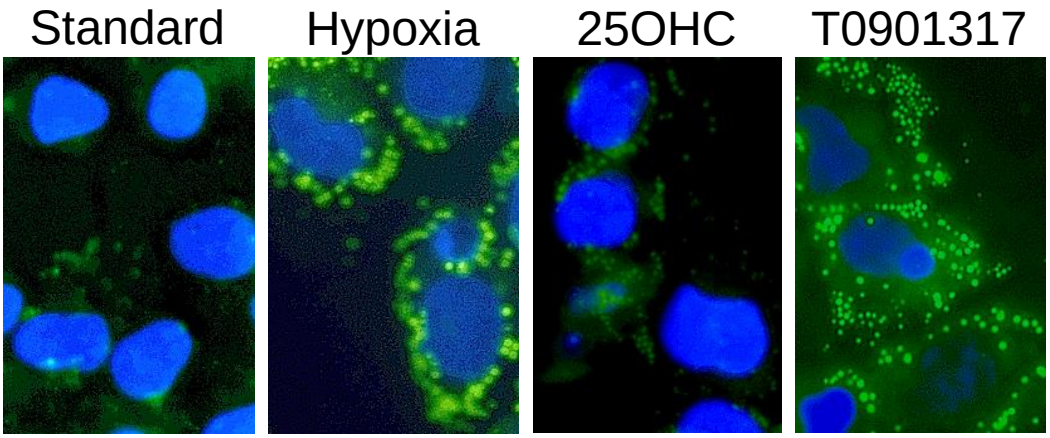
PHT cells (n=3) were treated with the indicated dose of T0901317 or 25OHC 24 hours after plating. LDH levels were measured at 48 and 72 hours after plating. Note that the non-parametric test of trend for LDH release was significant, with increasing doses of both ligands at both time points ($p<0.005$).

Figure 9 – Effects of oxysterols and T0901317 on progesterone secretion



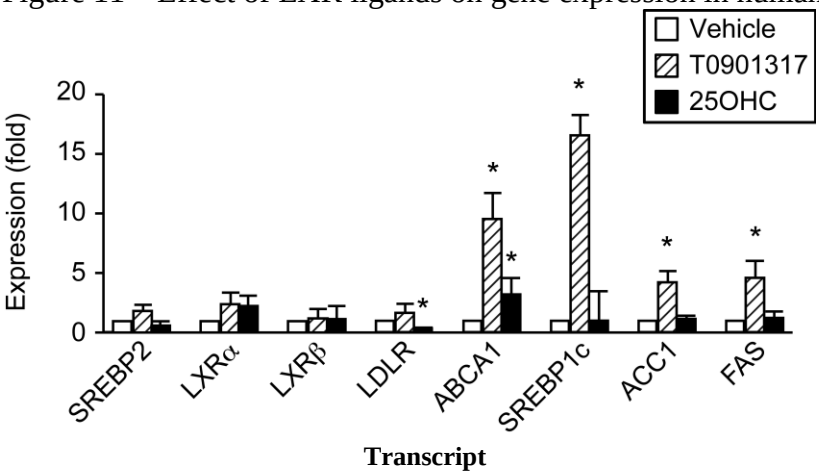
PHT cells were cultured at 21% O_2 and treated with the indicated dose of 25OHC or T0901317 24 hours after plating. Progesterone concentration in cell medium was measured 72 hours after plating.

Figure 10 – Effect of LXR ligands on lipid droplet formation



PHT cells were exposed to vehicle only, FiO_2<1%, 10 μM T0901317, or 10 μg/mL 25OHC prior to fixation and staining. Lipid droplets are stained with green BODIPY dye.

Figure 11 – Effect of LXR ligands on gene expression in human trophoblasts



RT-qPCR results (n=3, all experiments). *p<0.05 by Student's two-tailed t-test compared to cells exposed to vehicle only.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one

publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. The Expression and Localization of N-Myc Downstream-Regulated Gene 1 in Human Trophoblasts	Xiao-Hua Shi, Jacob C. Larkin, Baosheng Chen, Yoel Sadovsky	PLOS One	April, 2013	☐Submitted ☐Accepted ☒Published
2. NDRG1 Deficiency Attenuates Fetal Growth and the Intrauterine Response to Hypoxic Injury	Larkin J, Chen B, Shi XH, Mishima T, Kokame K, Barak Y, Sadovsky Y	Endocrinology	May, 2013	☐Submitted ☐Accepted ☒Published
3. The influence of ligand-activated LXR on primary human trophoblasts	Jacob Larkin, Sarah B. Sears, Yoel Sadovsky	Placenta	January, 2014	☒Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

I plan to submit multiple articles for publication. These articles will delineate the effect of hypoxia and oxidative stress on progesterone secretion in trophoblasts, the effect of hypoxia and oxidative stress on embryonic growth, placental gene expression, and fetal cholesterol accretion in mice, and the influence of LXR and SREBP2 manipulation on cholesterol trafficking in trophoblasts.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Larkin, Jacob C	Assistant Professor
PROJECT ROLE Principal Investigator	DISCIPLINE Maternal-Fetal Medicine
eRA COMMONS USER NAME LARKINJC	INSTITUTION University of Pittsburgh School of Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Chicago, Chicago, IL	B.A.	1992-1996	Philosophy
University of Pittsburgh, Pittsburgh, PA	M.D.	2000-2004	Medicine
University of Pittsburgh, Pittsburgh, PA		2009-2011	Clinical Research

A. Personal Statement

Mechanisms of placental injury and their clinical sequelae remain poorly understood and insufficiently investigated. The aim of our work is to link specific mechanistic pathways of placental dysfunction to relevant clinical outcomes and to identify therapeutic targets for amelioration of placental injury. This investigation merges expertise in the clinical assessment of fetal growth abnormalities with mechanistic studies of the effect of cholesterol oxidation on placental cholesterol homeostasis and fetal growth. Thus, we combine an insightful, novel and mechanistic approach to unanswered questions relevant to placental biology and fetal development with an intimate understanding of the clinical implications of fetal growth restriction and therapeutic limitations of current medical practice. Therapeutic targets identified through our work could represent novel interventions to influence placental injuries associated with fetal growth restriction.

B. Positions and Honors (in chronological order).

2004-2008: Resident in Obstetrics & Gynecology, Hospital of the University of Pennsylvania, Philadelphia, PA
2008-2011: Fellow in Maternal-Fetal Medicine, Magee-Womens Hospital, University of Pittsburgh, Pittsburgh, PA
2011-present: Assistant Professor, Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynecology, University of Pittsburgh School of Medicine, Pittsburgh, PA

PROFESSIONAL MEMBERSHIPS

2004-present American College of Obstetrics and Gynecology
2006-present Society for Maternal-Fetal Medicine
2007-present Society for Gynecologic Investigation

HONORS

1996 BA with General Honors, University of Chicago
2007 Departmental Research Grant to investigate angiogenic signaling in preeclampsia, University of Pennsylvania
2008 Resident Research Award, Dept. of Obstetrics & Gynecology, University of Pennsylvania
2008 Robert Rascoe Award for Excellence in Ambulatory Care, University of Pennsylvania
2010 AAOGF-SMFM Research Scholarship – 3-year, $300,000 grant to investigate the role of NDRG1 in placental injury
2011 Obstetrical Fellow Teaching Award, Dept. of Ob/Gyn, University of Pittsburgh
2012 Society for Maternal Fetal Medicine, Research Excellence Award
2013 APGO Excellence in Teaching Award, University of Pittsburgh

C. Selected peer-reviewed publications (in chronological order).

Srinivas SK, **Larkin J**, Sammel MD, Appleby D, Bastek J, Andrela CM, Ofori E, Elovitz MA. The use of angiogenic factors in discriminating preeclampsia: are they ready for prime time? *J Matern Fetal Neonatal Med* 2010;11:1294-300. PMID: 20482287

Larkin J, Speer P, Simhan H. A customized standard of large size for gestational age to predict intrapartum morbidity. *Am J Obstet Gynecol* 2011;204:499.e1-10. PMID: 21514553

Larkin J, Hill LM, Speer P, Simhan H. Risk of Morbid Perinatal Outcomes in Small-for-Gestational-Age Pregnancies: Customized Compared With Conventional Standards of Fetal Growth. *Obstet Gynecol,* 2012;119:21-7. PMID: 22183207.

Shi XH, **Larkin JC**, Chen B, Sadovsky Y. The expression and localization of N-myc downstream-regulated gene 1 in human trophoblasts. *PLoS One* 2013;8(9):e75473. PMID: 24066183

Larkin J, Chen B, Shi XH, Mishima T, Kokame K, Barak Y, Sadovsky Y. NDRG1 deficiency attenuates fetal growth and the intrauterine response to hypoxic injury. *Endocrinology* 2014; en20131425. [Epub ahead of print].

D. Research Support.

Ongoing

5K12HD063087 (Hogge, WA) 7/1/13 – 6/30/15
NIH-NICHD
Magee-Womens Basic and Translational Reproductive Health Training Program
In this application we seek to test the hypothesis that hypoxia and oxidative stress disrupt placental cholesterol trafficking and metabolism, and that LXR is a critical regulator of placental cholesterol homeostasis.