Video Article

Primary Endodermal Epithelial Cell Culture from the Yolk Sac Membrane of Japanese Quail Embryos

Han Jen Lin[1], Siou Huei Wang[1], Yu Hui Pan[1], Shih-Torng Ding[1]

[1]Department of Animal Science and Technology, National Taiwan University

Correspondence to: Shih-Torng Ding at sding@ntu.edu.tw

URL: http://www.jove.com/video/53624
DOI: doi:10.3791/53624

Keywords: Developmental Biology, Issue 109, Endodermal epithelial cell, embryo, Japanese quail, lipid utilization, primary cell culture, SOAT1, and yolk sac membrane

Date Published: 3/10/2016

Citation: Lin, H.J., Wang, S.H., Pan, Y.H., Ding, S.T. Primary Endodermal Epithelial Cell Culture from the Yolk Sac Membrane of Japanese Quail Embryos. *J. Vis. Exp.* (109), e53624, doi:10.3791/53624 (2016).

Abstract

We established an endodermal epithelial cell culture model (EEC) for studying the function of certain enzymes and proteins in mediating nutrient utilization by avian embryos during development. Fertilized Japanese quail eggs were incubated at 37 °C for 5 days and then yolk sac membranes (YSM) were collected to establish the EEC culture system. We isolated the embryonic endoderm layer from YSM, and sliced the membrane into 2 - 3 mm pieces and partially digested with collagenase before seeding in 24-well culture plates. The EECs proliferate out of the tissue and are ready for cell culture studies. We found that the EECs had typical characteristics of YSM *in vivo*, for example, accumulation of lipid droplets, expression of sterol O-acyltransferase and lipoprotein lipase. The partial digestion treatment significantly increased the successful rate of EEC culture. Utilizing the EECs, we demonstrated that the expression of SOAT1 was regulated by the cAMP dependent protein kinase A related pathway. This primary Japanese quail EEC culture system is a useful tool to study embryonic lipid transportation and to clarify the role of genes involved in mediating nutrient utilization in YSM during avian embryonic development.

Video Link

The video component of this article can be found at http://www.jove.com/video/53624/

Introduction

The major nutritional resource of the avian embryo is yolk, composed of 33% lipids, 17% protein, and 1% ash.[1] During embryonic development, the yolk sac membrane (YSM) grows from within the embryonic abdominal cavity and gradually covers the yolk surface. Beginning at embryonic day 2, gene expression associated with lipid metabolism and angiogenesis is gradually increased in YSM, and the YSM slowly develops villus-like projections.[8,9] These projections increase absorption of yolk nutrients to support embryonic development. The YSM is an extraembryonic tissue that contains three germ layers, endoderm, mesoderm and ectoderm.[14] The yolk sac ectoderm faces the albumen and links with the vitelline membrane to gently cover the yolk sac. The endodermal epithelial cells are faced directly toward the egg yolk and serve as nutrient utilization portals.[6] As the YSM expands, endodermal epithelial cell (EECs) can be divided by shape and functionality into two groups, area vitelline and area vasculosa.[7]

Area vitelline is composed of endodermal cells and is distant from the embryo; area vasculosa is composed of mesodermal cells and covers differentiated EECs with blood vessels and connective tissues. By embryonic day 5, the yolk is totally covered by ectoderm and endoderm of YSM and the vascular area has grown rapidly. The YSM absorbs, recomposes and releases lipids (as yolk-derived very low density lipoprotein) and proteins into the embryonic circulatory system.[9, 2] Therefore, we established a primary Japanese quail embryonic endodermal epithelial cell culture system, to study the mechanisms of lipid utilization in YSM during avian embryonic development.

Lipids such as triacylglycerol, lecithin, phospholipid and cholesterol ester (CE) are the primary energy sources for avian embryos. At the early stages of development, yolk lipids are composed of only 1.3% CE and it rises to 10-15% at mid-term of avian embryonic development [3, 11]. Cholesterol ester is synthesized from cholesterol by sterol O-acyltransferase 1 (SOAT1) in avian embryo YSM.[4]

The storage form of cholesterol is CE, CE is carried in lipoproteins, and lipoproteins are transported by circulation to tissues.[13] A week before hatch there is rapid growth of avian embryos. Approximately 68% of the remaining lipid contents in yolk are absorbed during this stage.[10] The mechanism by which yolk lipids are utilized can be clarified by an EEC research model. A chicken EEC culture protocol was established to achieve this research goal.[2, 9] However, due to the low success rate of tissue explants, an improved EEC cell culture procedure is needed to study the function of certain enzymes and proteins in mediating nutrient utilization by avian embryos during development.

Protocol

NOTE: This procedure is a modification of a chicken model culture protocol developed by Bauer *et al.* 2013 and Nakazawa *et al*., 2011. [2,9]

1. Prepare Healthy Embryonic Day 5 Embryos from Japanese Quail

1. Place 1 male and 3 female sexually mature Japanese quail together in the same cage. Supply feed and water *ad libitum*. Adjust the light in the animal room to 14 hr of light and 10 hr of darkness.
2. Collect fertilized eggs in plastic bags every day in the afternoon and keep them in a 16 °C refrigerator to retard the growth rate of embryos.
3. After collecting a sufficient number of eggs within a two-week period, incubate the fertilized eggs in a 37 °C incubator with good ventilation for five days.

2. Prepare Basal Medium and Wash Solutions

1. Use DMEM/ F-12 medium (adjusted to pH 7.2) as the growth medium, and supplement it with 10% new born calf serum (NBCS) and 1% Pen-Strep Ampho. Solution (PSA; Penicillin, 10^5 units/L; Streptomycin, 100 mg/L; Amphotericin B, 0.25 mg/L).
2. Prepare phosphate buffered saline (PBS) solution at a 10x concentration for storage; use 1x PBS solution (containing NaCl, 137.93 mM; KCl, 2.667 mM; KH_2PO_4, 1.471 mM; Na_2HPO_4-$7H_2O$, 8.06 mM; adjust to pH 7.2) to wash and moisten the yolk sac membrane while separating the endoderm from egg yolk and albumin.
3. Use DMEM medium (adjusted to pH 7.2) to re-suspend endoderm pellets.

3. Collection of Primary EECs from Japanese Quail YSMs

1. Incubate the collected eggs for 5 days (step 1.3). Examine the eggs by an egg candler to confirm normal embryonic development.
   NOTE: On embryonic day 5, the boundary of YSM mesoderm is very clear and the 3 embryonic layers are less tightly-linked together, so it is easy to obtain endoderm. Only use the eggs showing normal capillary circulation development for YSM collections.
2. Clean the eggshell carefully using fresh water and 75% ethanol. Open the eggshell from the air cell position by a pair of scissors, gently peel out and collect the whole YSM by forceps and place in a 10 cm culture dish filled with 37°C 1x PBS.
3. Using a pair of scissors remove the embryo, yolk and egg-white, and gently wash the YSM with 1x PBS three times and place YSM in PBS solution in a 10 cm culture dish.
4. Use forceps and a pair of scissors to remove the ectoderm from YSM. Use 2 forceps, one to firmly hold the endodermal cell layer, and the other to hold the capillary mesoderm.
5. Under a dissecting microscope, pull apart and separate the endoderm from the edge of the mesoderm in the direction towards the embryo. Collect the light-yellow endoderm in 50 ml centrifuge tubes using a dropper and place in 15 ml of growth medium in a 37 °C water bath until all tissue collections are completed.

4. Digestion of the Endoderm Slices by Collagenase Digestion

1. Freshly dissolve 6.5 units of type IV collagenase in 10 ml of DMEM.
2. Centrifuge the 50 ml tubes (3.4) at 130 x g for 3 min at RT. Aspirate the medium and place all the endoderm in a 6 cm culture dish using a pipette. Add 1 ml collagenase solution to this culture dish.
3. Slice the endoderm collected from 2 quail embryos with a pair of curved scissors until the size of each slice is approximately 2 to 3 mm. Use a dropper, collect all the slices into a 50 ml centrifuge tube along with 9 ml of fresh collagenase solution.
4. Digest the tissue with collagenase for 30 min in a 37 °C shaking water bath at 175 rpm. This partial digestion with collagenase is used to improve the explant cell proliferation.
5. Centrifuge at 130 x g for 3 min at RT and gently remove the supernatant fraction using a pipette. Wash the pellet by suspension in 20 ml DMEM and remove the supernatant fraction after 130 x g centrifugation for 3 min.
6. Re-suspend the pellet with 12 ml of growth medium (DMEM/F12 with 10% NBCS and 1% PSA) and seed gently and equally into a 24-well plate (each well with 500 µl cell solution).
7. Incubate tissue explants at 37 °C in 5% CO_2 in air for 2 days to allow cells to proliferate out of the tissues.
8. Incubate cells for another 2 days and characterize for cell viability by a cell viability assay protocol.[15]
   NOTE: Living cells maintain a reducing environment within the cytosol. Resazurin, the active ingredient, is a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent. When added to cell cultures, resazurin is reduced to resorufin in the cytosol by mitochondrial enzymes. Resorufin is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the culture medium surrounding cells.
9. Perform endoderm epithelial cell total RNA isolation, reverse transcription and quantitative real-time PCR of marker gene mRNA expression as described. [16,17]
   1. Use reverse transcriptase PCR to detect SOAT1 mRNA (sense: 5'-GAAGGGGCCTATCTGGAACG-3'; antisense: 5'-ATCTGCACGTGACATGACCA-3'; PCR product = 168 bp) as a specific marker for the EEC cells. Use β-Actin mRNA (sense: 5'-TGGTGAAGCTGTAGCCTCTC-3'; antisense: 5'- GTGATGGACTCTGGTGATGG-3'; PCR product = 151 bp) as a housekeeping gene and internal control.
   2. Use gel electrophoresis to detect the product size of RT-qPCR. Prepare 1% agarose gel, and mix 10 µl qPCR product with 2 µl loading dye. Load 10 µl mixture into each well in the 1% agarose gel, and run the gel by 100V for 30 min.

Representative Results

In order to achieve the goal of establishing a consistent and useful cell model, we need to extend and stabilize the proliferation rate and performance of avian EECs. We compared direct incubation of the endoderm with no enzyme digestion with endoderm partially digested with proteolytic enzymes, such as collagenase or collagenase plus 0.6 U of dispase. Dispase is an amino-endopeptidase that hydrolyzes the N-terminal peptide bonds of non-polar amino acid residues. Whereas protease digestion treatments increased cell proliferation compared with no digestion (**Figure 1**), cell growth was not markedly different between the two enzyme treatments after five days incubation (**Figure 2**). However, the collagenase treatment resulted in a better growth curve and showed that EECs proliferated steadily for 6-9 days (**Figure 2**). Thus, partial collagenase digestion improved growth of EECs from the ex-vivo explants and is recommended to obtain sufficient and functional EEC.

After 4 days incubation, EECs had an adipocyte-like appearance detected after formalin fixation and Oil-Red O staining (**Figure 3)**, suggesting correct physiological characteristics of the cultured cells.

We hypothesized that cyclic adenosine monophosphate (cAMP) is a transcription regulatory agent, the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; to decrease degradation of cAMP) and the adenyl cyclase activator, forskolin (to increase synthesis of cAMP) both stimulated *SOAT1* mRNA expression after 24 hr treatment (**Figure 4**). These treatments also increased the expression of SREBP2, a cholesterol synthesis regulatory transcription factor, and forskolin enhanced the expression of perilipin-2 (PLIN2), the surface protein marker of lipid droplets (**Figure 5**). The EEC mRNA profiles for lipogenic genes quantified by real-time PCR are similar to adipocytes (**Figure 5**).

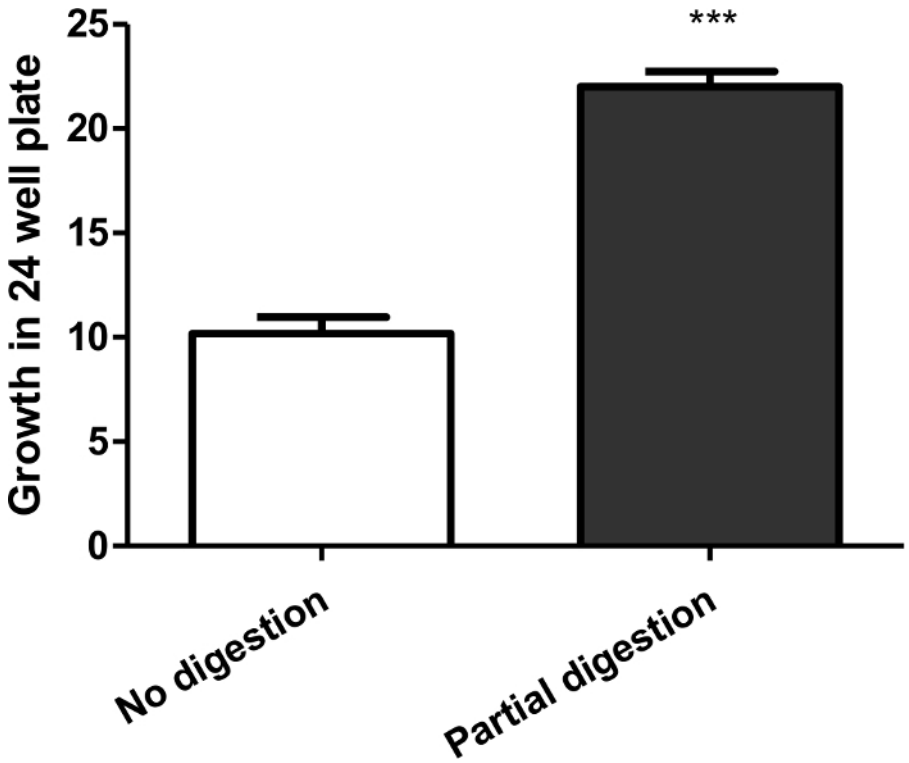


Figure 1. Effect of Partial Collagenase Digestion on Endodermal Epithelium Cells. Tissue explants were incubated for 2 days to allow cells to proliferate out of the tissue. We observed and calculated the numbers of the successfully grown EECs. Numbers were calculated for cells with no digestion and cells partially digested with collagenase; both were grown in 24-well plates. The Y-axis is well number of successfully proliferated EECs in cell culture plate of 24-well. Data were presented as Mean ± SEM. Analysis were determined by paired t test. P< 0.0001. Please click here to view a larger version of this figure.

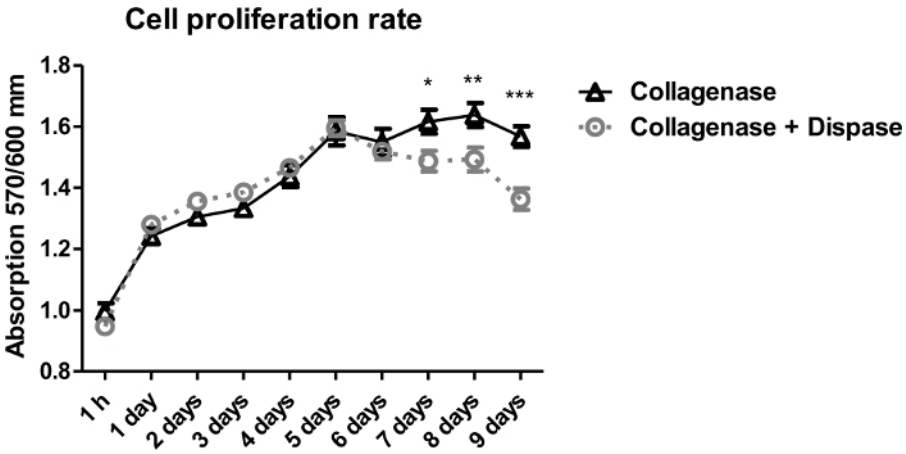
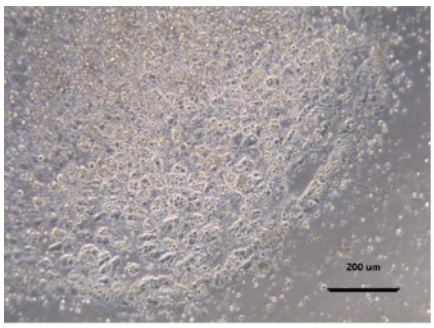
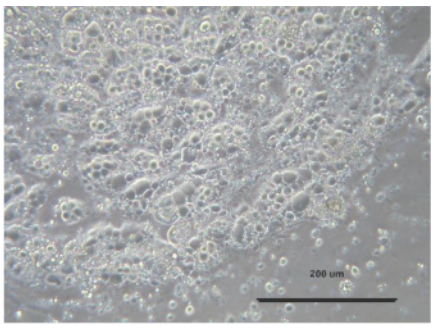


Figure 2. The Cell Growth Performance of EECs After Partial Enzyme Digestion. The proliferation rate was detected by cell viability assay. The assay contained resazurin, a blue compound that has weak fluorescence and resazurin is reduced to resorufin upon entering cells. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. Viable cells were detected by absorbance at 570 nm with normalization at 600 nm. Data were presented as Mean ± SEM. Analysis were determined by two-way ANOVA with Bonferroni post-test (collagenase group n=10; collagenase + dispase group n=11). * P < 0.05, ** P<0.01, *** P<0.001. Please click here to view a larger version of this figure.





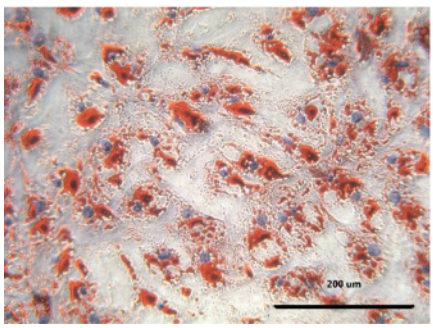


Figure 3. The Morphology of Cultured EECs. After 4 days incubation, EECs had an adipocyte-like appearance detected after formalin fixation and Oil-Red O staining. From left to right: EEC's in bright field with 100X magnification, in bright field with 200X magnification and in 200X magnification after staining with Oil-red O and hematoxylin. The dark spot on the left of the far left figure is the partially digested endoderm (membrane). Scale bar represents 200 µm. Please click here to view a larger version of this figure.

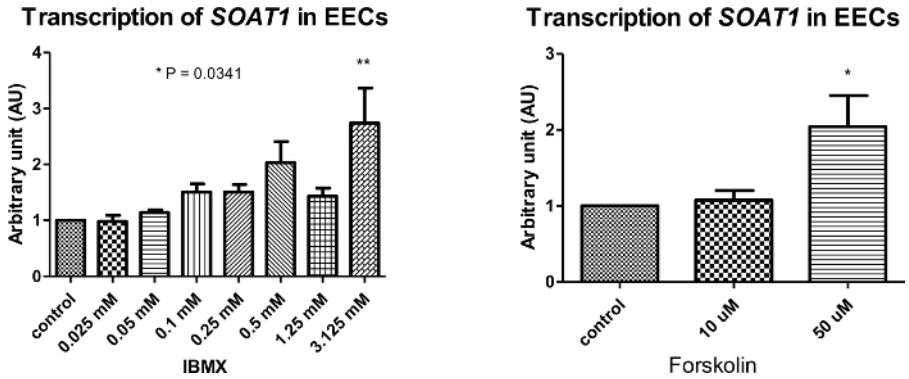


Figure 4. The SOAT1 Transcription of EECs Treated with cAMP Activators. We treated EECs with increasing concentrations of IBMX or forskolin at day 5 for 24 hr. We extracted mRNAs from treated cells and converted the RNA into cDNA by reverse transcription PCR. We analyzed transcription levels by real-time quantitative PCR. β-actin was used as the housekeeping gene. Data were presented as Mean ± SEM. Analyses were by one-way ANOVA with Tukey's post-test (n = 6) (*P≤ 0.05, ** P≤ 0.01). IBMX is a competitive nonselective phosphodiesterase inhibitor which raises intracellular cAMP, activates PKA, inhibits TNF-alpha and leukotriene synthesis, reduces inflammation and innate immunity and is a nonselective adenosine receptor antagonist. Forskolin is a ubiquitous activator of eukaryotic adenylyl cyclase, commonly used to raise levels of cAMP. Please click here to view a larger version of this figure.

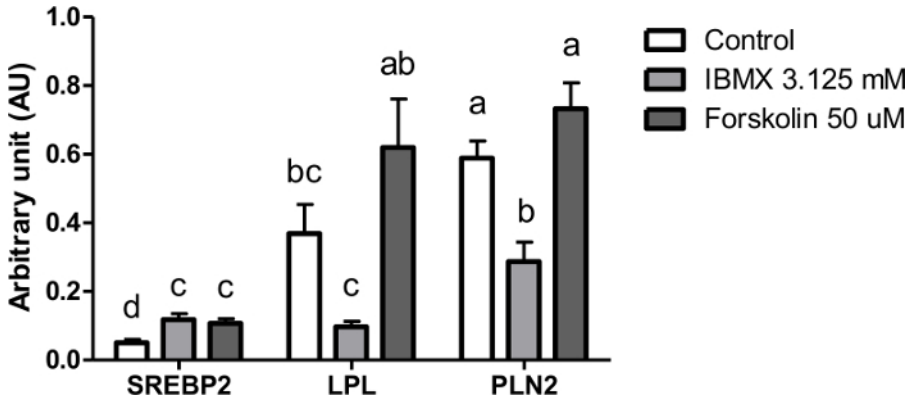


Figure 5. The mRNA Expressions of EEC's Grown for Five Days, Then Treated with cAMP Activators for 24 hr. The cDNA's in **Figure 4** were used to analyze gene expression levels by real-time quantitative PCR. β-actin was used as the housekeeping gene and served as the internal control. Data were presented as Mean ± SEM. Analyses were by one-way ANOVA with Tukey's post-test (n≤ 6) to compare treatments to the control. SREBP2, sterol regulatory element-binding protein 2; LPL, lipoprotein lipase; PLIN2, perilipin-2. Please click here to view a larger version of this figure.

Discussion

Because the previous culture system has only limited success, a better culture system is needed. The Japanese quail YSM endoderm requires treatment with proteolytic enzymes such as collagenase to loosen the cell-cell junctions to achieve better growth performance in the ex-vivo explants. Our data show that cell numbers from partial digestion treatment were greater than from the undigested tissue culture after seeding for 2 days (**Figure 1**). Therefore, the partial proteolytic digestion is a critical step to improve cell yield from the YSM.

The critical modification in the current culture protocol is the partial proteolytic digestion. This treatment greatly enhanced the cell yield and the successful rate of obtaining live EECs. The digestion time is very critical because over-digestion will separate cells from the tissue and cells will not attach to the plate, whereas under-digestion resulted in less cell yield from the tissue explants. We found that 30 to 40 min digestion using the conditions described above is optimal.

The current protocol requires high technical training to obtain EECs. The time point of collecting EECs is restricted, for example, we only collect EECs at embryonic day 5. The reason is that as avian embryos grow, the connections between layers in the yolk sac membrane are more complex and it decreases the successful rate of collecting EECs. The protocol can be used to obtain EECs of early stage embryos but not late stage embryos. It limits the possibility of direct research on the late stage EECs.

The expression of SOAT1 and lipoprotein lipase, and lipid accumulation are essential characteristics of the YSM [4,5,11], especially the EECs. In the current study, SOAT1, LPL, and perilipin 2 (PLN2) mRNA were expressed highly in the EECs, suggesting the culture system can generate cells with the correct physiological characteristics. The current technique produced a large quantity of primary EECs. When we used the previous described procedure[9], the success rate of EEC culture was approximately 60% in cell culture plates of 24-well, whereas the current protocol generated EECs at nearly 100% success rate.

Yolk sac membrane is the main tissue to absorb proteins, lipids, and cholesterol from yolk, thus functioning to transfer nutrients from yolk to embryos during development. The main enzyme for cholesterol ester formation is SOAT1.[11] Because SOAT1 increases dramatically in the late

stages of avian embryonic development when massive cholesterol esterification occurs[4], we used *SOAT1* mRNA expression as the marker gene for evaluating the characteristics of cultured EECs. We demonstrated that this primary EEC culture system produced expected physiological characteristics. Therefore, the EEC culture system can be a valuable tool to study embryonic lipid transportation and to clarify the role of genes involved in mediating nutrient utilization in YSM during avian embryonic development.

Disclosures

There is no competing financial interests or other conflicts of interest. We did not get any funding from any private company.

Acknowledgements

Funding for the development of current protocol is particularly supported by “Aim for the Top University Plan” of the National Taiwan University, Taiwan (grant ID-104R350144), as well as the Ministry of Science and Technology of Taiwan (grant ID: MOST 104-2313-B-002-039-MY3). We especially thank the Center for Biotechnology of National Taiwan University for providing an animal room and laboratory space for the current study.

References

1. Abeyrathne, E.D., Lee, H.Y., Ahn, D.U. Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents--a review. *Poult Sci.* **92** (12), 3292 - 3299 (2013).
2. Bauer, R., Plieschnig, J.A., Finkes, T., Riegler, B., Hermann, M., Schneider, W.J. The developing chicken yolk sac acquires nutrient transport competence by an orchestrated differentiation process of its endodermal epithelial cells. *J Biol Chem.* **288** (2), 1088 - 1098 (2013).
3. Ding, S.T., Nestor, K.E., Lilburn, M.S. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks of age. *Poult Sci.* **74** (2), 374 - 382 (1995).
4. Ding, S.T., Lilburn, M.S. The developmental expression of acyl-coenzyme A: cholesterol acyltransferase in the yolk sac membrane, liver, and intestine of developing embryos and posthatch turkeys. *Poult Sci.* **79** (10), 1460 - 1464 (2000).
5. Ding, S.T., Lilburn, M.S. The ontogeny of fatty acid-binding protein in turkey (Meleagridis gallopavo) intestine and yolk sac membrane during embryonic and early posthatch development. *Poult Sci.* **81** (7), 1065 - 1070 (2002).
6. Kanai, M., Soji, T., Sugawara, E., Watari, N., Oguchi, H., Matsubara, M., Herbert, D.C. Participation of endodermal epithelial cells on the synthesis of plasma LDL and HDL in the chick yolk sac. *Microsc Res Tech.* **35** (4), 340 - 348 (1996).
7. Mobbs, I.G. McMillan, D.B. Structure of the endodermal epithelium of the chick yolk sac during early stages of development. *Am J Anat.* **155** (3), 287 - 309 (1979).
8. Mobbs, I.G., McMillan, D.B. Transport across endodermal cells of the chick yolk sac during early stages of development. *Am J Anat.* **160** (3), 285 - 308 (1981).
9. Nakazawa, F., Alev, C., Jakt, L.M., Sheng, G. Yolk sac endoderm is the major source of serum proteins and lipids and is involved in the regulation of vascular integrity in early chick development. *Dev Dyn.* **240** (8), 2002 - 2010 (2011).
10. Noble, R.C., Cocchi, M. Lipid metabolism and the neonatal chicken. *Prog Lipid Res.* **29** (2), 107 - 140 (1990).
11. Shand, J.H., West, D.W., McCartney, R.J., Noble, R.C., Speake, B.K. The esterification of cholesterol in the yolk sac membrane of the chick embryo. *Lipids.* **28** (7), 621 - 625 (1993).
12. Speier, J.S., Yadgary, L., Uni, Z., Wong, E.A. Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. *Poult Sci.* **91** (8), 1941 - 1949 (2012).
13. Walzem, R.L., Hansen, R.J., Williams, D.L, Hamilton, R.L. Estrogen Induction of VLDLy Assembly in Egg-Laying Hens. *J Nutr.* **129** (2), 467S - 472S,ISSN: 0022-3166, (1999).
14. Yoshizaki, N., Soga, M., Ito, Y., Mao, K.M., Sultana, F., Yonezawa, S. Two-step consumption of yolk granules during the development of quail embryos. *Dev Growth Differ.* **46** (3), 229 - 238 (2004).
15. *alamarBlue Cell Viability Assay Protocol.* Source: https://www.lifetechnologies.com/us/en/home/references/protocols/cell-and-tissue-analysis/cell-profilteration-assay-protocols/cell-viability-with-alamarblue.html#4 (2008).
16. Lin, H.Y., Chen, C.C., Chen, Y.J., Lin, Y.Y., Mersmann, H.J., Ding, S.T. Enhanced Amelioration of High-Fat Diet-Induced Fatty Liver by Docosahexaenoic Acid and Lysine Supplementations. *Biomed Res Int.* **2014** (1), 310981 (2014).
17. Lin, Y.Y. et al. Adiponectin receptor 1 regulates bone formation and osteoblast differentiation by GSK-3β/β-Catenin signaling in mice. *Bone.* **64,** 147 - 154 (2014).

