PHYSIOLOGY AND REPRODUCTION

Finite cell lines of turkey sperm storage tubule cells: ultrastructure and protein analysis

Neil C. Talbot,[1] Katina V. Krasnec, Wesley M. Garrett, Amy E. Shannon, and Julie A. Long

USDA, ARS, BARC, Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA

ABSTRACT Cell lines of turkey sperm storage tubule (SST) epithelial cells were established. Turkey SSTs were dissected from freshly obtained uterovaginal junction (UVJ) tissue and placed in explant culture on various substrates and media. Primary cultures of SST epithelium only survived and grew from SST explants that were cultured on inactivated Sandoz inbred strain, thioguanine- and ouabain-resistance (STO) mouse feeder-cell layers in 12% fetal bovine serum-supplemented Dulbecco's Modified Eagle Medium mixed 1:1 with F12 nutrient mixture. Three independent primary colonies gave rise to 3 finite cell lines, SST-1, -2, and -3, which were continuously cultured for 8 to 16 passages at 1:3 passage ratios over a period of 3 to 4 mo. The cells were passaged by pretreatment with Y27632 and dissociation with Accutase. The SST cells grew as tightly knit monolayers on top of the feeder cells at a slow rate (approximately 96 h doubling time) at a medium pH of approximately 6.9. Lipid vacuoles were visible by light microscopy in the cells particularly at the periphery of growth. Transmission electron microscopy revealed the cells to be a polarized epithelium with apical microvilli and to have lateral tight-junction-like unions and associated desmosomes. Numerous secretory vesicles filled the upper portion of the cells' cytoplasm, and nuclei and other major organelles such as mitochondria, rough endoplasmic reticulum, and Golgi apparatus were distributed somewhat lower in the cytoplasm. The secretory vesicles resembled mucin secretory vesicles. Proteomic analysis by mass spectroscopy of the conditioned medium of the cells, and of the cells themselves, showed the cell lines did not secrete large amounts of any particular protein, and the analysis confirmed their epithelial character. In conclusion, the SST-derived cell lines resembled the mucus-secreting cells found in the epithelium lining the UVJ of the turkey's reproductive tract.

Key words: cell, culture, sperm, storage, turkey

INTRODUCTION

Female turkeys (hens), like chicken hens and other female birds, are able to "store" sperm in their reproductive tracts after copulation, thus allowing the continuous fertilization of eggs as they are produced over a period of weeks (Bobr et al., 1964a,b; Bakst, 1998; Sasanami et al., 2013). The sperm are stored where the turkey hen's vagina meets the shell gland (uterus), the uterovaginal junction (**UVJ**), in specialized invaginations of the surface epithelium termed sperm storage tubules (**SST**). Two salient biological questions about turkey SSTs remain to be fully understood: how they maintain sperm viability over time and how they release sperm.

Cell and molecular biology studies being conducted by multiple laboratories around the world seek to answer these fundamental questions, and in vitro models of SST could contribute to these efforts. Presently, in vitro models of the SSTs of turkeys or chickens do not exist. Numerous examples of the short-term cell culture or organ culture of chicken UVJ, infundibulum, magnum, and shell gland (uterus) epithelium have been published, and they encompassed a wide array of scientific investigations (Ashizawa et al., 1976; Ashizawa and Nishiyama, 1983; Seaver et al., 1984; Sanders and McKnight, 1985; Jung-Testas et al., 1986; Jung et al., 2011; Kasperczyk et al., 2012; Ariyadi et al., 2013; Mork et al., 2014). The report of Kasperczyk et al., (2012)

detailed several approaches to the primary culture, in a monolayer format, of chicken magnum or infundibulum tissue after various mechanical cell harvesting methods combined with collagenase digestion. Also of note is the paper by Jung et al., (2011), where the effects of hormone treatments on magnum epithelial cells were examined, because the authors established the cells as finite cell lines and sustained their continuous culture for 25 passages for cells from juvenile hens (10 wk old) and for at least 6 passages from mature hens (30 wk old). In contrast to the chicken, apparently, no reports exist for the turkey concerning the culture of oviductal tissues other than a published method for the isolation of individual SSTs that was proposed to be useful as the starting point for in vitro studies (King et al., 1999).

Given the limited number of reports on avian UVJ in vitro models, and the absence of any SST primary cell culture models or cell lines, we undertook to culture and characterize turkey SST epithelial cells as continuous cultures, i.e., as finite epithelial cell lines.

MATERIALS AND METHODS

Turkey UVJ Isolation and SST Cell Culture

Care and treatment of all turkeys used in this study were approved by the Institutional Animal Care and Use Committee of the Beltsville Animal Research Center, United States Department of Agriculture. For UVJ tissue isolation, non-inseminated, commercial turkey hens in egg production for 4 wk were euthanized by intravenous sodium pentobarbital injection (100 to 150 mg sodium pentobarbital/kg body weight) at 34 wk of age to recover reproductive tissue. The UVJ tissue was dissected from the reproductive tract as previously described (Bakst, 1992). Briefly, the uterus and vagina were dissected from the reproductive tract together, and the extraneous connective tissue was removed from the UVJ before cutting longitudinally with scissors to expose the mucosa containing the SSTs. Tissue sections approximately 4×4 mm in size of the surface epithelium and its immediate underlying stromal layer were cut from turkey UVJ tissue to begin SST cell cultures.

Turkey SST cell lines were established and grown in high glucose Dulbecco's Modified Eagle Medium (**DMEM**; Hyclone, GE HealthCare Life Sciences, Logan, UT) mixed 1:1 with F12 nutrient mixture (Cat. no. N4888; Sigma-Aldrich, St. Louis, MO) and supplemented with 12% v/v fetal bovine serum (**FBS**; Atlanta Biologicals, Lawrenceville, GA) and 2 mM L-glutamine (**12% DMEM/F12**). Primary cultures of SST cells were begun by transferring the UVJ tissue sections to a 35-mm plastic petri dish containing 12% DMEM/F12 medium. Individual SSTs were dissected from the UVJ tissue sections using 27 G hypodermic needles while under observation through a binocular dissection microscope (Nikon, Inc., Tokyo, Japan). Single SSTs were transferred to various culture substrates

and media conditions using a mouth-controlled, finely drawn, sterile, Pasteur pipet. All primary SST cell cultures were established as explants cultures in 4-well culture plates (Nalgen Nunc International, Rochester, NY) placed in cell culture incubators (Sanyo Scientific, Osaka, Japan) with a 92.5% air/7.5% CO_2 humidified atmosphere at 37°C. The cultures' media were exchanged with fresh 12% DMEM/F12 medium every 2 to 3 d. Culture substrates included a thin layer of polymerized collagen type I (PureCol, Advanced Matrix, Inc., San Diego, CA) prepared as previously described (Talbot et al., 2010a), a thin-layer (1:40 dilution in 12% DMEM/F12) of Matrigel (Becton/Dickson, Lincoln Park, NJ), and mouse fibroblast feeder layers. The preparation of feeder cells was as previously described using 8 Krad of gamma radiation to inactivate Sandoz inbred strain, thioguanine- and ouabain-resistance (**STO**) mouse embryonic fibroblasts (CRL 1503, American Type Culture Collection, Rockville, MD) or primary cultures of CF-1 strain mouse fibroblasts (Talbot et al., 2012). Stock STO and CF-1 cells were grown in 10% DMEM high glucose (Hyclone) supplemented with 4 mM L-glutamine (Hyclone) and penicillin/streptomycin (Gibco, Thermo Fisher Scientific Inc, Grand Island, NY). Feeder layers consisted of irradiated STO or CF-1 cells plated at 2.5×10^4 cells/cm^2 and maintained with weekly feeding of 10% DMEM until use for the culture of SST cells.

Sperm storage tubule primary cultures and the cell lines derived from them were propagated in secondary culture by pretreatment of the cells with Y27632 (10 μM; Stemgent, San Diego, CA), a selective inhibitor of Rho-associated, coiled-coil containing protein kinase (**ROCK**), for 30 to 60 min (Watanabe et al., 2007). Either trypsin-ethylenediaminetetraacetic acid [trypsin-EDTA (0.05% trypsin/0.2 g/l EDTA•4Na); Hyclone, ThermoFisher] or Accutase dissociation solution (Sigma-Aldrich) was used to dissociate the SST cells from one another and their culture substrate. For routine passage (**P**), cells were washed 2 times with Dulbecco's phosphate buffered saline (**PBS**) without Ca^{++} and Mg^{++} (Gibco, ThermoFisher) and washed 1 time with Accutase leaving a thin layer of Accutase (approximately 100 μL) in the T12.5 T-flask to dissociate the cells after incubation for 5 to 10 min at 37°C. The released cells were suspended in 12% DMEM/F12 medium with Y27632 (10 μM), and the clumps of cells were triturated by repeated serological pipet aspirations to a nearly single-cell suspension and plated on fresh STO feeder layers (T12.5 flasks) at 1:1, 1:2, or 1:3 split ratio. The following day, after the SST cells had attached, the medium was exchanged to 12% DMEM/F12 medium without Y27632.

Other cell culture reagents or physicochemical conditions (e.g., medium pH and height) that were tested on the SST cultures, but not necessarily reported on (below) due to a lack of beneficial proliferative effect, included the following: normal adult chicken serum (Sigma); basic fibroblast growth factor, epidermal

growth factor, and leukemia inhibitory factor obtained from R&D Systems, Minneapolis, MN; progesterone, estradiol, butyrate, dexamethasone, and forskolin obtained from Sigma.

For cryopreservation, SST cell monolayers were dissociated with Accutase as described above. The dissociated cells were resuspended in ice cold 92% FBS/8% dimethyl sulfoxide (Sigma) and distributed in aliquots of 1 mL to cryovials (Nunc, Denmark). The cryovials were placed in a styrofoam box and the box placed into a –80°C freezer for slow freezing. The frozen cryovials were transferred to a liquid nitrogen dewar (Locator Jr; ThermoFisher) for long-term storage.

Periodic Acid-Schiff Staining

Periodic acid-Schiff (**PAS**) staining of cultured SST cells for mucin detection was done using a PAS staining kit (PolyScience, Inc., Warrington, PA) according to the manufacturer's instructions. The cultured SST cells were fixed in situ (T12.5 tissue culture flask culture) with ultrapure 4% formaldehyde (Polysciences, Inc.) in Hank's buffered saline solution (ThermoFisher, Gibco) for 20 min prior to PAS staining. Mucin-positive cells display a magenta color.

Transmission Electron Microscopy

Transmission electron microscopy (**TEM**) sample preparation and photomicroscopy were done with assistance of JFE Enterprises, Brookville, MD. For SST cell culture fixation, the medium from T12.5 flask cultures was removed and 2 mL of 2.5% glutaraldehyde at approximately 37°C was added to each flask for 30 min fixation at room temperature followed by 30 min fixation at approximately 8°C. The 2.5% glutaraldehyde was removed and the flask was washed in ice cold Millonig's buffer (Millonig, 1964) and stored under 2 mL of Millonig's buffer at approximately 8°C. Tissue samples of UVJ (approximately 4×8 mm pieces) that had most of their underlying connective tissue removed were taken from 4 euthanized turkey hens that were in egg production—2 with artificial insemination and 2 non-inseminated birds. After fixation with 2.5% glutaraldehyde (2 h), approximately 1 mm square sections of UVJ/SST tissue were prepared for TEM by dissection with the aid of a dissection microscope (Nikon) and 27 G hypodermic needles. Tissue samples and SST cell line monolayers were post-fixed with 1% osmium tetroxide and stained with 2% uranyl acetate. Samples were dehydrated in ethanol and placed in propylene oxide prior to embedding in Epon 812. SST cell line monolayers were embedded a second time on their underside after release of the first embedding from the T-flask's plastic bottom. Ultrathin plastic sections were prepared and stained with lead citrate for examination with a Ziess EM10 CA transmission electron microscope.

Analysis of SST Cell line Conditioned Medium by 1-Dimensional Gel Electrophoresis and Mass Spectrometry

Nearly confluent 3 wk post-passage T12.5 flasks cultures of SST-1 and -2 cells (P4 and P3, respectively) and 2 wk post-passage SST-2 cultures (3 T12.5 flasks at P6) were washed 4 times with serum-free (**SF**) DMEM/F12 medium and each culture was covered with 2 mL of the SF medium. After 72 h of incubation in the SF medium, the serum-free conditioned medium (**SFCM**) was collected into sterile centrifuge tubes and centrifuged at approximately 1,000 × g to pellet any dead cell debris. The supernatants were collected and combined from the P3 and P4 SST-1 and -2 flasks and stored frozen at –75°C. The supernatants from the 3 flasks of P6 SST-2 cultures were similarly combined and frozen. The SFCM samples were concentrated approximately 75-fold using 10 Kda molecular weight cut-off Vivaspin 500 concentrators (Sartorius Corp., Bohemia, NY), and their constituent proteins were separated by 1-dimensional gel electrophoresis. The resulting protein bands were cut out, trypsin-treated, and extracted, and their constituent proteins were identified by mass spectroscopy as previously described (Talbot et al., 2007; Caperna et al., 2013).

Analysis of SST Cell Line Cellular Proteins by 2-Dimensional Gel Electrophoresis and Mass Spectrometry

A T12.5 culture of SST-1 at passage 15 was washed with 0.25 M sorbitol solutions 3 times and immediately frozen at –75°C. Upon thawing, the SST-1 cells (and STO feeder-layer cells) of the flask were lysed with urea lysis buffer and processed for 2-dimensional (2D) gel electrophoresis as previously described (Talbot et al., 2010b). The separated proteins were made visible by colloidal Coomassie Blue staining (NuSep Ltd, Australia), and protein spots were punched out of the gel, trypsin digested, and the resulting peptides analyzed on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) as previously described (Talbot et al., 2010b).

RESULTS

Freshly dissected, whole turkey SSTs were cultured as primary explant cultures on various cell culture substrates in 12% DMEM/F12 medium. The substrates included bare tissue cultureware plastic, "thin-layer" polymerized type I collagen coating, thin-layer Matrigel coating, and feeder layers of irradiated STO mouse fibroblasts. In all cases, at least some individual SST attached to each of the substrates over a 24 h period in culture. The cells of the SST slowly spread out over each substrate in 3 to 5 d. Those SST cells cultured on bare tissue culture plastic slowly enlarged because of
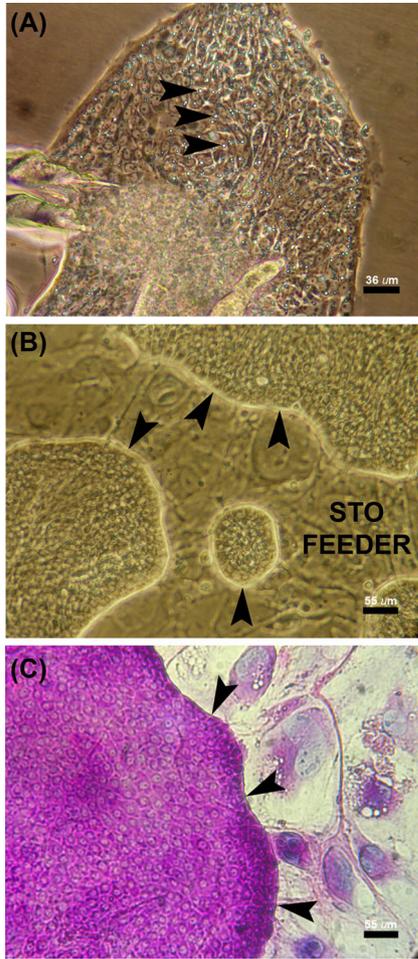
Figure 1. Phase-contrast light micrographs of SST cells in culture. (**A**) Primary outgrowth (72 h) of SST explants cultured on polymerized collagen type I substrate. Arrowheads indicate small lipid droplets in the cells. (B) SST-1 cell line at passage 12 on STO feeder-cell substrate. Arrowheads indicate the edges of SST-1 colony monolayers expanding across and on top of the STO feeder cells. (C) Periodic acid Schiff positive staining of passage 13 SST-2 cells in monolayer on STO feeder cells. Arrowheads indicate the edge of the SST-2 monolayer. SST, sperm storage tubule; STO, Sandoz inbred strain, thioguanine- and ouabain-resistance.

the colony's individual cells spreading out flatter and flatter over a period of weeks in primary culture until the cells lost any semblance of normal epithelial morphology, i.e., cuboidal cells. The cells of SSTs plated on polymerized collagen or Matrigel did not flatten out and retained an epithelial morphology for a period of at least 2 to 3 wk, but these primary colonies did not noticeably proliferate. Only the SST explant cultures on STO feeder cells appeared to be continuously dividing over the first 2 to 3 wk of primary culture.

In the first few days of culture 2 cell types could be discerned in most of the primary colonies—multiciliated cells and non-ciliated cells (Figure 1A). In all the colonies, the ciliated cells, if present, were a small minority population. Presumably the ciliated cells were from UVJ lumenal epithelial cells that remained attached to the proximal end of the SSTs because the SST epithelium was found to be composed of non-ciliated

cells in its entirety (see ultrastructure analysis below; Schuppin et al., 1984). Thus, despite every effort to dissect only the SST epithelium, some of the UVJ epithelium, consisting of ciliated epithelial cells and mucus-secreting epithelial cells, must have contaminated the SST cells established in the explant cultures. The cilia of the multiciliated cells beat continuously for the entirety of the primary culture period (2 to 3 wk with refeeding every 48 h), but their numbers did not increase in the primary explant colony outgrowths. Also, the ciliated cells did not survive, or, alternatively, they did not regenerate their cilia, after the first secondary passage of the cultures using trypsin-EDTA as the dissociating agent.

Secondary passage of 3 SST primary outgrowths was pursued to establish cell lines. The SST-1 and SST-2 cell lines were established from whole-dissected SSTs that in their primary outgrowth had contaminating ciliated cells present. The SST-3 cell line was distinct in being cultured from the "tip-end" distal portion of an SST, to avoid any contamination of the SST-3 cell line by UVJ ciliated or mucus-secreting cells, i.e., epithelial cells lining the lumen of the UVJ. Secondary passage of SST primary outgrowths also revealed that dissociation of the primary SST cells from the underlying substrate and themselves resulted in catastrophic cell blebbing, as with the dissociation of primary pig epiblast cells or human embryonic stem cells from one another (Talbot and Garrett, 2001; Watanabe et al., 2007). Pretreatment of the SST cultures with ROCK inhibitor, Y27632, prevented the catastrophic blebbing and enabled the SST cells to survive and reattach in new flasks. Secondary culture of the cells also confirmed that only the SST cells grown on STO feeder layers continued to divide to increase their numbers. These non-ciliated, closely knit epithelial cells were dividing and doubled in their total numbers of cells several times. The primary outgrowths were expanded to T12.5 flasks containing STO feeder cells and finite cell lines were established that were capable of being passaged up to at least 8 times and being expanded to millions of cells (Figures 1B and 2). After 10 to 12 passages, however, the cell lines growth slowed with the cell population apparently becoming senescent. The 3 SST cell lines, each established from an independent turkey reproductive tract, were readily cryopreserved and were frozen at the third passage level for later culture and analysis.

While the SST cells grew on the STO feeder cells, their growth was relatively slow, and, therefore, several culture modifications were empirically tested to try to increase their growth rate and thus improve their usefulness as an in vitro model. Potential enhancement of growth rate was assessed by microscopic observations. Adult chicken serum (10% v/v) in place of FBS in the DMEM/F12 medium did not improve the growth of the SST-1 cells. Amendment of the FBS-containing 12% DMEM/F12 medium with estradiol (100 nM), progesterone (20 ng/mL), epidermal growth factor (100 ng/mL), leukemia inhibitory
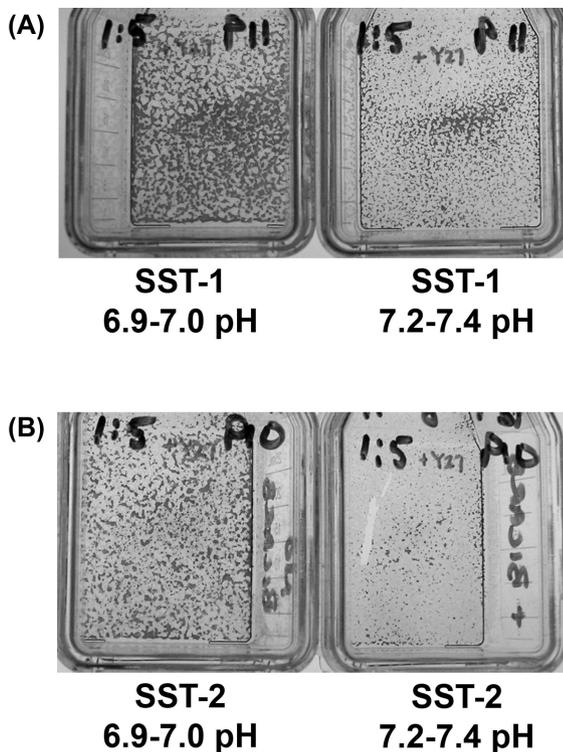
Figure 2. Enhanced growth of SST cell lines at reduce pH. The SST culture flasks were simultaneously fixed and stained with a solution of 0.125% Coomassie Blue R-250 (Sigma) in 50% methanol/10% acetic acid prior to photography. The hundreds/thousands of growing SST colonies are visible on the bottom of each flask allowing comparison of culture at pH 6.9–7.0 vs. pH 7.2–7.4. SST, sperm storage tubule

factor (10 ng/mL), or basic fibroblast growth factor (8 ng/mL) for a 2-wk period did not obviously change of the rate of growth of the SST-2 cell line. Reducing the height of the medium in the flasks, to increase the oxygen content of the medium at the level of the cells (Taylor and Camalier, 1982; Pettersen et al., 2005; Oze et al., 2012), had no observable effect on growth rate. The opposite condition of culturing in a low oxygen atmosphere (5% v/v) also did not appear to improve the growth rate. However, growth rate of the SST cells was increased at a lower, slightly acidic pH (Figure 2). The 12% DMEM/F12 medium typically had a pH of approximately 6.9 when incubated at $37°C$ in a 7.5% CO_2 atmosphere. This was a result of the F12 component of the medium not containing any sodium bicarbonate. Therefore, our "standard" 12% DMEM/F12 medium had additional sodium bicarbonate added to it to adjust the pH of the medium to the "normal" physiological level of pH 7.3. Thus, without the added sodium bicarbonate the cells grew much better (Figure 2). Figure 2 also illustrates that the cells usually grew to an approximate 50% of confluent condition over a 7 to 10 d culture period after each passage.

Transmission electron microscopic cell ultrastructure examinations of the SST-2 and SST-3 cell lines showed that the cells grew as either a monolayer of roughly cuboidal cells or, in at least some areas, as multilay-

ers of cells (Figure 3A and B). The cells were joined at their apical aspect by tight-junction-like junctions and laterally by desmosomes. Adjacent cells had numerous interdigitating cytoplasmic folds at their lateral surfaces (Figure 3A and B), and there was not a well-developed basement membrane associated with the bottom of the cells. The cells were polarized in character with apical microvilli and numerous secretory vesicles typical of mucus-containing vesicles in the upper half of the cells, often amassed under the apical cell membrane (Figure 3A and C). Positive PAS staining of the SST-1 and -2 cell sheet was consistent with the vesicles being mucus-containing vesicles (Figure 1C). No cilia were observed on the cells. Cell organelles that were well represented in the cells' cytoplasm were mitochondria with flat cristae, Golgi apparatus, usually arranged around the nuclei of the cells, rough endoplasmic reticulum, and relatively small lipid vacuoles. Some of these ultrastructural features contrasted with those of the in vivo SST cells (Figure 4). Notably, the in vivo SST cells usually displayed a prominent, supranuclear lipid droplet (Figure 4B and C) and their densely packed apical microvilli were elaborating vesicles from their tip ends (Figure 4D). Also, the in vivo SST cells did not appear to contain the mucus vacuoles that were such a prominent feature of the SST-1 and -2 cells (Figure 4B and D).

Proteomic analysis of the SST cell lines' SFCM was conducted by 1-dimensional gel electrophoresis followed by mass spectrophotometric analysis of the separated proteins (Supplementary Data Spreadsheet S1 and Table 1). A total of 52 proteins were identified. The 35 proteins identified as turkey (Meleagris gallopavo) were mostly cytoplasmic or cell membrane constituent proteins common to most cells. Of possible interest, however, were the avian cytokeratins types 2, 18, and 19, and annexin A8 as epithelial cell markers and a potential sperm/oviduct interaction protein, respectively. Also, the anterior gradient protein 2 homolog (**AGR-2**) was of possible significance because of its association with mucus-secreting cells. Clusterin, matrilysin, galectin-2, zonadhesin-like protein, and macrophage migration inhibitory factor (**MIF**) were also found and may be significant because of their previous associations with reproductive tract/sperm biology. The other proteins identified in the SFCM probably originated from lysed cells in the culture, particularly the mouse STO feeder cells, which die slowly over time.

Cellular proteins of SST-1 cells were identified from a 2D gel and they represented the most abundant proteins in the cell lysate sample since no fractionations or differential enrichments were performed (Supplementary Data Set S2 and Table 1). From the 112 protein spots harvested and analyzed, a total of 157 proteins were identified, 93 as turkey and 60 as mouse (deriving from the STO feeder cells). Most of the identified turkey proteins were cytoplasmic or cytoskeletal proteins common to all cell types. As stated above for the SFCM proteins, the cytokeratins (1, 2,
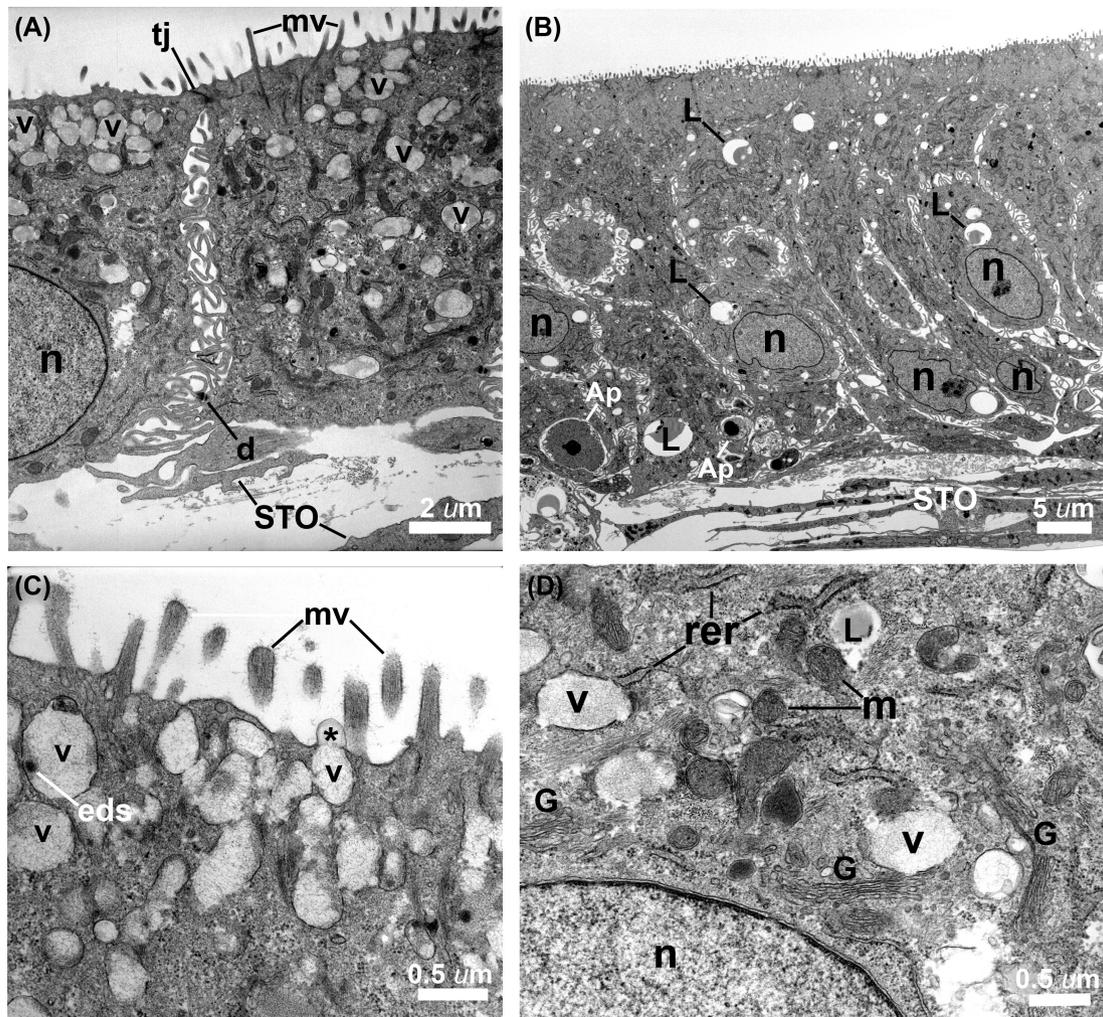
Figure 3. Transmission electron microscopy of turkey SST cell lines. (A) SST-2 cells growing as monolayer on the STO feeder cells and displaying extensive cytoplasmic membrane lateral interdigitations, apical tight-junction-like adhesions, and basolateral desmosome connections. (B) SST-3 cells growing as a more than 1 cell thick layer. Note lipid vacuoles (L) and apoptotic bodies (Ap). (C) SST-3 cell showing mucus vesicles (v) with typical eccentric dense spherule (eds) and imminent secretion of mucus at apical cytoplasmic membrane (*). (D) Various organelles identified in SST-2 cells. Abbreviations: n = nucleus, d = desmosome, eds = eccentric dense spherule, G = Golgi apparatus, L = lipid vacuole, m = mitochondria, mv = microvilli, v = mucus vesicle, rer = rough endoplasmic reticulum. SST, sperm storage tubule; STO, Sandoz inbred strain, thioguanine- and ouabain-resistance.

8, 10, 18, 19, and cochleal) and annexins (A2, A4, A5, A8, A11) are of interest as epithelial cell markers and potential sperm/oviduct interaction proteins, respectively. Heat shock protein 90 alpha (HSP90A), maestro heat-like repeat-containing protein family member 7, and galectin-3 were also found and may be significant because of their previous associations with reproductive tract/sperm biology.

DISCUSSION

The study demonstrates the culture of turkey SST cells as primary cultures and as continuous cultures of finite cell lines, i.e., a homogenous culture of cells that could be passaged several times while maintaining the cells' essential character. Four observations were notable about the cells or cell lines, 1) the SST cells were sensitive to dissociation with trypsin-EDTA and required pretreatment with ROCK inhibitor Y27632 to prevent cell lysis, 2) the SST cells were dependent on mesenchymal feeder cells (mouse fibroblasts) for their continuous culture, 3) the SST cells grew better at a relatively acidic pH, i.e., relative to a normal physiological pH of 7.3, and 4) the SST cells did not maintain their in vivo ultrastructural character of having a very large perinuclear lipid vacuole(s), and instead appeared to become a mucus-secreting epithelium. The proteomic examinations of the cells and their conditioned medium confirmed their turkey identity and their epithelial nature, and revealed some findings that could be linked with how the SSTs function in maintaining the viability of sperm in the turkey hen's reproductive tract for weeks.

The better propagation of the SST cell lines at relatively acidic pH (approximately 6.9) may reflect the pH of the turkey UVJ which was reported to range from pH 6.93 min after oviposition to pH 7.24, 8
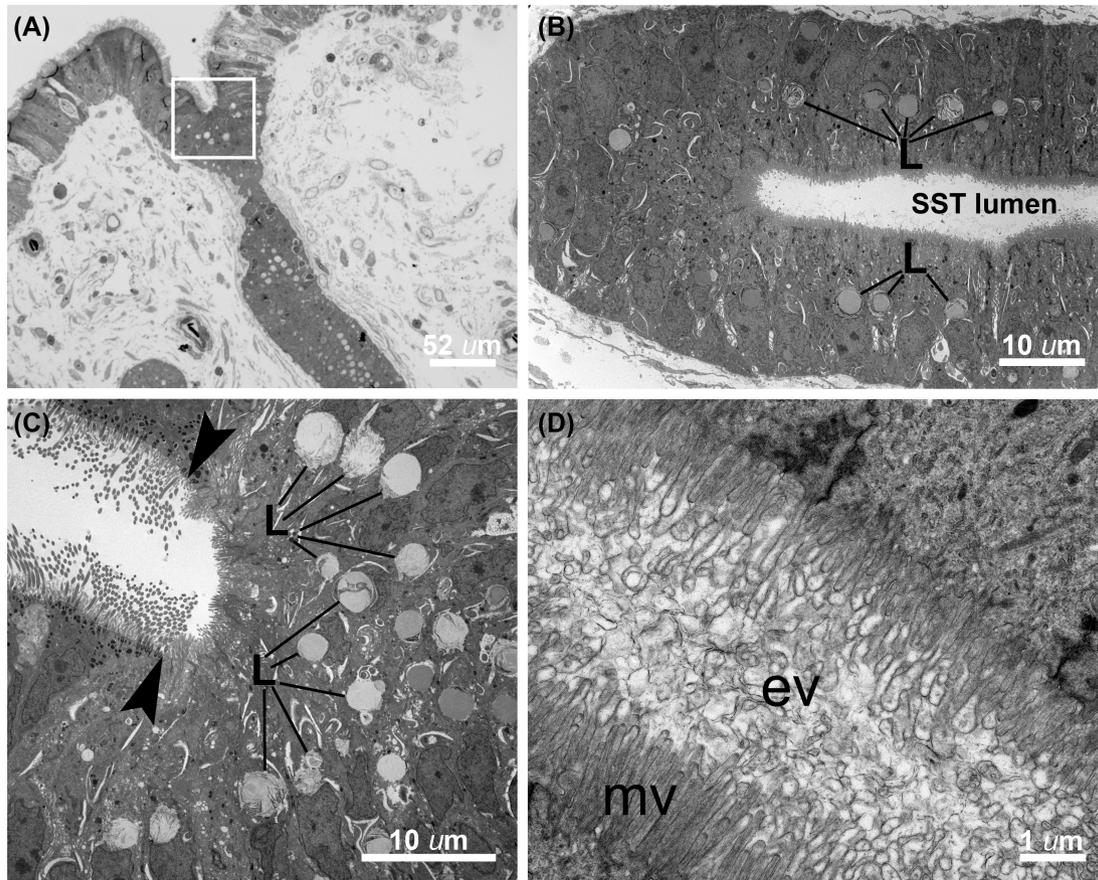
Figure 4. Transmission electron micrography of in vivo turkey UVJ/SST. (A) Longitudinal section of an SST and its connection with the UVJ (delineated by the box). (B) Distal end portion of the SST from (A) showing the SST lumen formed by the columnar epithelium with dense apical cell surface microvilli and supranuclear lipid vacuoles (L). (C) Higher magnification image of boxed area in (A) with arrowheads indicating the beginning of the SST where the alternating ciliated cells and mucus-secreting cells of the UVJ stop. Numerous lipid vacuoles (L) in the SST cells are indicated. (D) SST from an inseminated turkey hen showing active release of extracellular vesicles (ev) from the tips of the epithelial cells' microvilli (mv). SST, sperm storage tubule; UVJ, uterovaginal junction.

to 12 h postoviposition (Bakst, 1980). Similarly, the chicken UVJ pH ranged from 6.92 to 7.18 (Bakst, 1980). Also, in the chicken, mating with insemination was found to upregulate numerous genes involved with pH regulation (Atikuzzaman et al., 2015). It has also been shown that the motility of sperm from domestic birds is effectively inhibited by lowering the pH in vitro (Holm and Wishart, 1998), and this has been proposed as a possible mechanism for making sperm quiescent in vivo in the oviduct's SSTs (Holm et al., 1996).

The SST cells in culture did not maintain some hallmark morphological features, e.g., the large perinuclear lipid droplet and microvillar release of extracellular vesicles observed in in vivo SST cells (Figures 3 and 4; Friess et al., 1978; Schuppin et al., 1984). Moreover, the cells of the SST cell lines adopted a non–in vivo-like secretory phenotype, i.e., mucus vacuole production and secretion (Figures 3 and 4; Schuppin et al., 1984). Our electron microscopy examinations of UVJ tissues from 4 turkey hens in egg production, 2 inseminated and 2 non-inseminated, confirmed past studies of chicken and turkey SST cell morphology. After sampling mul-

tiple SSTs in their longitudinal aspect, it was unequivocal that the columnar epithelial cells comprising the turkey SSTs were non-ciliated, as previously reported (Schuppin et al., 1984), had a pronounced lipid vacuole over most nuclei, also as previously reported (Schuppin et al., 1984), and appeared to be non-secretory in character—also as previously described for the SSTs of birds and reptiles (Schuppin et al., 1984; Bakst, 1998; Sever and Hamlett, 2002).

The proteomic examinations of the SST cells' conditioned medium did not indicate a large secretion of any particular protein from the cultured cells. The turkey proteins found in the SST SFCM were disappointing in being mostly cytoplasmic proteins common to most cells (Table 1). Similarly, the 2D-gel survey of the cellular proteins from a crude cell lysate of SST-1 cells resulted in the identification of mostly common cellular proteins. However, taken together, the proteomic analysis revealed some proteins of interest, either as marker proteins or for their potential biological function. Two groups of proteins identified as turkey that were of possible note were cytokeratins (types 1, 2, and 6A; Table 1) and annexins (A2, A4, A5, and A8;

Table 1. Selected proteins identified from SST cell lines

Protein name	Gene	Species	Accession number	Protein molecular weight	Unique peptides	Spectral count
			SST cell lysate proteins			
Annexin A2	ANXA2	Meleagris gallopavo	XP˙01,947,4821.1	39 kDa	7	7
Annexin A4	ANXA4	Meleagris gallopavo	XP˙01,072,1503.1, XP˙01,072,1504.1	36 kDa	7	7
Annexin A5	ANXA5	Meleagris gallopavo	XP˙01,070,8237.1	36 kDa	10	10
Annexin A8-like protein 1	ANXA8L1	Meleagris gallopavo	XP˙01,071,2766.1, XP˙01,947,3160.1, XP˙01,947,3162.1, XP˙01,947,3163.1, XP˙01,947,3164.1, XP˙01,947,3165.1, XP˙01,947,3166.1	33 kDa	11	12
Annexin A11	ANXA11	Meleagris gallopavo	XP˙01,071,2435.1	52 kDa	7	7
Anterior gradient protein 2	AGR2	Meleagris gallopavo	XP˙01,071,1044.1	20 kDa	4	4
Heat shock protein 90-alpha	HSP90AA1	Meleagris gallopavo	XP˙01,071,0229.1	84 kDa	1	1
Keratin, type I cytoskeletal 18	KRT18	Meleagris gallopavo	XP˙01,946,7106.1	48 kDa	11	12
Keratin, type I cytoskeletal 19-like	KRT19	Meleagris gallopavo	XP˙0,032,13149.2	46 kDa	10	10
Keratin, type I cytoskeletal 20	KRT20	Meleagris gallopavo	XP˙0,032,13156.1	49 kDa	5	6
Keratin, type II cytoskeletal 8	KRT8	Meleagris gallopavo	XP˙01,072,6168.1, XP˙01,072,6169.1	52 kDa	2	2
Keratin, type II cytoskeletal cochleal	K2CO	Meleagris gallopavo	XP-0,032,06045.1	54 kDa	20	20
			SST conditioned medium proteins			
Annexin A8-like protein 1	ANXA8L1	Meleagris gallopavo	XP˙01,071,2766.1, XP˙01,947,3160.1, XP˙01,947,3162.1, XP˙01,947,3163.1, XP˙01,947,3164.1, XP˙01,947,3165.1, XP˙01,947,3166.1	33 kDa	1	1
Anterior gradient protein 2	AGR2	Meleagris gallopavo	XP˙01,071,1044.1	20 kDa	2	2
Clusterin	CLU	Meleagris gallopavo	XP˙01,070,6357.1	52 kDa	2	3
Keratin, type I cytoskeletal 18	KRT18	Meleagris gallopavo	XP˙01,946,7106.1	48 kDa	5	5
Keratin, type I cytoskeletal 19-like	KRT19	Meleagris gallopavo	XP˙0,032,13149.2	46 kDa	1	1
Keratin, type II cytoskeletal cochleal	K2CO	Meleagris gallopavo	XP˙0,032,06045.1	54 kDa	4	4
macrophage migration inhibitory factor	MIF	Meleagris gallopavo	XP˙01,947,6467.1	13 kDa	1	1
Matrilysin	MMP7	Meleagris gallopavo	XP˙0,032,03531.1	30 kDa	2	2
Zonadhesin-like	ZAN	Meleagris gallopavo	XP˙0,032,09168.2	100 kDa	1	1

Table 1). Cytokeratins confirmed the epithelial nature of the cells (Moll et al., 1982). The annexins could be of significance because annexin-A1 and -A2 were detected on the surface epithelium of the avian oviduct and in gene expression analysis of the chicken egg gland (Chailley and Pradel, 1992; Yang et al., 2007). Also, annexin-A1, -A2, -A4, and -A5 are involved with sperm/oviductal cell adhesion and release in other species (reviewed in Talevi and Gualtieri, 2010).

Other proteins identified that are of potential interest in SST function included ARG-2, HSP90A, **MIF**, clusterin, matrilysin, galectin-3, and zonadhesin-like protein. AGR-2 can be a secreted protein, and it is strongly expressed in mucus-secreting tissues, which is consistent with the observation of the numerous mucus vesicles in the SST cell line cells (Park et al., 2009; Li et al., 2012a). AGR-2 may also be significant because it was found to have specific expression in the avian oviduct (Kim et al.,

2007), its expression interrelated with estrogen and the estrogen receptor (Vanderlaag et al., 2010; Bu et al., 2013), and it has been shown to be involved with cell growth, tissue repair, and tissue regeneration (Wodziak et al., 2016; Zhu et al., 2017). These characteristics may contribute to SST development, maintenance, and function, particularly if estrogen is important in the cytodifferentiation of SSTs as it is in the differentiation of the tubule glands of the magnum (Palmiter and Wrenn, 1971; Pescatore and Marquez, 1977; Yoshimura et al., 2000; Holm and Ridderstråle, 2002; Das et al., 2006a).

HSP90A can be a secreted protein and it causes changes in the expression and function of LDL receptor related protein 1, matrix metallopeptidase 2, and tyrosine kinases (Li et al., 2012b), all target proteins with reported links to sperm/oviduct maintenance and function (Ashizawa et al., 1998; Birkenmeier et al., 1998; Leśniak-Walentyn and Hrabia, 2016, 2017). In vitro sperm viability in cattle and pigs was correlated with HSP90 expression and content (Huang et al., 1999; Wang et al., 2014). Other reports describe HSP90 interaction with the progesterone receptor in the chicken oviduct (Renoir et al., 1993), which, therefore, connects HSP90 with SST sperm storage and release (Yoshimura et al., 2000; Ito et al., 2011). Exosome-mediated secretion of HSP90 (Takeuchi et al., 2015) supports its potential involvement in the hypothetical support of sperm in the SSTs by the SST epithelium's robust microvillar exosome (extracellular vesicle) production (Figure 4; Schuppin et al., 1984; Bakst and Bauchan, 2015).

The remaining proteins, MIF, clusterin, galectin-3, matrilysin, maestro heat-like repeat-containing protein family member 7, and zonadhesin-like protein are also related to reproductive tract/sperm biology, either directly or indirectly, and in various ways. Briefly, MIF treatment of human sperm was found to inhibit sperm capacitation and decrease sperm motility (Carli et al., 2007). Both these effects are proposed to be fundamental to the function of the turkey's SSTs in their storage of sperm (Bakst, 1998; Sasanami et al., 2013). Clusterin is a chaperone protein that is, among other things, expressed in the male mammalian reproductive tract in association with sperm, where it may function in improving cell survival (Law and Griswold, 1994; Ammar and Closset, 2008). Similarly, galectin-3 was found in seminal fluid of various mammals and is also expressed in the female mammalian reproductive tract (Jones et al., 2010; Nio-Kobayashi, 2017). Matrilysin (matrix metalloproteinase-7) has been shown to be expressed in the glandular epithelium of the chicken oviduct (Leśniak-Walentyn and Hrabia, 2016). Maestro heat-like repeat-containing protein family members were found to be constituents of the chicken egg shell membrane (Makkar et al., 2015). Finally, zonadhesin-like protein is related to zonadhesin, a protein component of sperm that functions in mammalian sperm–zona pellucida binding (Lea et al., 2001). The detection of zonadhesin-like protein in the conditioned medium of the SST cells may therefore be related to avian sperm–egg interaction.

The cells of the SST-derived cell lines established and partially characterized here do not strictly resemble their in vivo counterparts, i.e., non-secretory epithelial cells with prominent supranuclear lipid vacuoles and microvilli shedding extracellular vesicles (Schuppin et al., 1984; Bakst et al., 1994). The cultured SST cells are polarized epithelial cells with apical microvilli, and, while they do contain some lipid vacuoles, their primary feature is their apparent secretion of mucus from the numerous vesicles arrayed under their apical cell membranes. Since contamination of the SST cell lines with the mucus-secreting cells of the UVJ seems unlikely— since the SST-3 cell line was carefully derived from the distal end of an SST, where no mucus-secreting UVJ cells would be present—there seemingly must be another explanation for our results. One hypothesis could be that the in vivo SST epithelium evolutionarily derives from the non-ciliated, mucus-secreting epithelium of the UVJ, and, along with the evolution of the tubule structure, the cells have evolved to downregulate mucus production and instead store more lipid, presumably for the nutritional support of sperm while they are resident in the SSTs (Bakst et al., 1994). The artificial environment of in vitro culture may then have caused the resumption of the mucus-secreting phenotype for unknown reasons. Further investigations of culture environments and methods need to be tested in the hope of finding in vitro conditions that result in retention of the in vivo characteristics of the SST cells. If in vivo phenotypic fidelity can be achieved, it is likely that the SST cell lines would be a useful in vitro model for investigations of the mechanisms of in vivo SST function.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online

REFERENCES

Ammar, H., and J. L. Closset. 2008. Clusterin activates survival through the phosphatidylinositol 3-kinase/Akt pathway. J. Biol. Chem. 283:12851–12861.

Ariyadi, B., N. Isobe, and Y. Yoshimura. 2013. Induction of mucin expression by estrogen and lipopolysaccharide in the lower oviductal segments in hens. Poult. Sci. 92:3205–3213.

Ashizawa, K., M. Higashio, and Y. Tsuzuki. 1998. Effects of tyrosine kinase inhibitor on the motility and ATP concentrations of fowl spermatozoa. Mol. Reprod. Dev. 49:196–202.

Ashizawa, K., and H. Nishiyama. 1983. Prolonged survival of fowl spermatozoa in the oviducal tissues in organ culture. Br. Poult. Sci. 24:27–32.

Ashizawa, K., H. Nishiyama, and T. Nagae. 1976. Effects of oviducal cells on the survival and fertilizing ability of fowl spermatozoa. Reproduction. 47: 305–311.

Atikuzzaman, M., R. Mehta Bhai, J. Fogelholm, D. Wright, and H. Rodriguez-Martinez. 2015. Mating induces the expression of immune- and pH-regulatory genes in the utero-vaginal junction containing mucosal sperm-storage tubuli of hens. Reproduction. 150:473–483.

Bakst, M. R. 1980. Chicken and turkey oviductal pH at known times postoviposition. Poult. Sci. 59: 2793–2796.

Bakst, M. R. 1992. Observations on the turkey oviductal sperm-storage tubule using differential interference contrast microscopy. Reproduction. 95: 877–883.

Bakst, M. R. 1998. Structure of the avian oviduct with emphasis on sperm storage in poultry. J. Exp. Zool. 282:618–626.

Bakst, M. R., and G. Bauchan. 2015. Apical blebs on sperm storage tubule epithelial cell microvilli: their release and interaction with resident sperm in the turkey hen oviduct. Theriogenology. 83:1438–1444.

Bakst, M. R., G. Wishart, and J.-P. Brillard. 1994. Oviducal sperm selection, transport, and storage in poultry. Poult. Sci. Rev. 5:117–143.

Birkenmeier, G., E. Usbeck, A. Schäfer, A. Otto, and H. J. Glander. 1998. Prostate-specific antigen triggers transformation of seminal alpha2-macroglobulin (alpha2-M) and its binding to alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein (alpha2-M-R/LRP) on human spermatozoa. Prostate. 36:219–225.

Bobr, L. W., F. W. Lorenz, and F. X. Ogasawara. 1964a.Distribution of spermatozoa in the oviduct and fertility in domestic birds. I. Residence sites of spermatozoa in fowl oviducts. J. Reprod. Fertil 8:39–47.

Bobr, L. W., F. X. Ogasawara, and F. W. Lorenz. 1964b. Distribution of spermatozoa in the oviduct and fertility in domestic birds. II. Transport of spermatozoa in the fowl oviduct. J. Reprod. Fertil. 8:49–58.

Bu, H., M. R. Schweiger, T. Manke, A. Wunderlich, B. Timmermann, M. Kerick, L. Pasqualini, E. Shehu, C. Fuchsberger, A. S. Cato, and H. Klocker. 2013. Anterior gradient 2 and 3–two prototype androgen-responsive genes transcriptionally upregulated by androgens and by oestrogens in prostate cancer cells. FEBS J. 280:1249–1266.

Caperna, T. J., A. E. Shannon, W. M. Garrett, T. G. Ramsay, L. A. Blomberg, and T. H. Elsasser. 2013. Identification and characterization of a nuclear factor-kappa B-p65 proteolytic fragment in nuclei of porcine hepatocytes in monolayer culture. Domest. Anim. Endocrinol. 45:154–162.

Carli, C, P. Leclerc, C. N. Metz, and A. Akoum. 2007. Direct effect of macrophage migration inhibitory factor on sperm function: possible involvement in endometriosis-associated infertility. Fertil. Steril. 88:1240–1247.

Chailley, B., and L. A. Pradel. 1992. Immunodetection of annexins 1 and 2 in ciliated cells from quail oviduct. Biol. Cell. 75: 45–54.

Das, S. C., N. Nagasaka, and Y. Yoshimura. 2006. Changes in the expression of estrogen receptor mRNA in the utero-vaginal junction containing sperm storage tubules in laying hens after repeated artificial insemination. Theriogenology 65: 893–900.

Friess, A. E., F. Sinowatz, K. H. Wrobel, and R. Scklek-Winnisch. 1978. The uterovaginal sperm host glands of the quail (Coturnix coturnix japonica). Cell Tissue Res. 191:101–114.

Holm, L., and Y. Ridderstråle. 2002. Development of sperm storage tubules in the quail during sexual maturation. J. Exp. Zool. 292:200–205.

Holm, L., Y. Ridderstråle, and P-G. Knutsson. 1996. Localisation of carbonic anhydrase in the sperm storing regions of the domestic hen oviduct. Acta Anat (Basel). 156:253–260.

Holm, L., and G. J. Wishart. 1998. The effect of pH on the motility of spermatozoa from chicken, turkey and quail. Anim. Reprod. Sci. 54:45–54.

Huang, S. Y., Y. H. Kuo, W. C. Lee, H. L. Tsou, Y. P. Lee, H. L. Chang, J. J. Wu, and P. C. Yang. 1999. Substantial decrease of heat-shock protein 90 precedes the decline of sperm motility during cooling of boar spermatozoa. Theriogenology. 51:1007–1016.

Ito, T., N. Yoshizaki, T. Tokumoto, H. Ono, T. Yoshimura, A. Tsukada, N. Kansaku, and T. Sasanami. 2011. Progesterone is a sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology. 152:3952–3962.

Jones, J. L., S. Saraswati, A. S. Block, C. F. Lichti, M. Mahadevan, and A. B. Diekman. 2010. Galectin-3 is associated with prostasomes in human semen. Glycoconj. J. 27:227–236.

Jung, J. G., T. S. Park, J. N. Kim, B. K. Han, S. D. Lee, G. Song, and J. Y. Han. 2011. Characterization and application of oviductal epithelial cells in vitro in gallus domesticus. Biol. Reprod. 85:798–807.

Jung-Testas, I., T. Garcia, and E. E. Baulieu. 1986. Steroid hormones induce cell proliferation and specific protein synthesis in primary chick oviduct cultures. J. Steroid Biochem. 24:273–279.

Kasperczyk, K., A. Bajek, R. Joachimiak, K. Walasik, A. Marszalek, T. Drewa, and M. Bednarczyk. 2012. In vitro optimization of the Gallus domesticus oviduct epithelial cells culture. Theriogenology. 77:1834–1845.

Kim, N. S., Y. N. Shen, T. Y. Kim, S. J. Byun, I. S. Jeon, and S. H. Kim. 2007. Expression of AGR-2 in chicken oviduct during laying period. J. Biochem. Mol. Biol. 40:212–217.

King, L. M., J. P. Brillard, M. R. Bakst, and A. M. Donoghue. 1999. Isolation of sperm storage tubules from the uterovaginal junction mucosa of the turkey. Poult. Sci. 78:1044–1047.

Law, G. L., and M. D. Griswold. 1994. Activity and form of sulfated glycoprotein 2 (Clusterin) from cultured sertoli cells, testis, and epididymis of the rat. Biol. Reprod. 50:669–679.

Lea, I. A., P. Sivashanmugam, and M. G. O'Rand. 2001. Zonadhesin: characterization, localization, and zona pellucida binding. Biol. Reprod. 65:1691–1700.

Leśniak-Walentyn, A., and A. Hrabia. 2016. Involvement of matrix metalloproteinases (MMP-2, -7, -9) and their tissue inhibitors (TIMP-2, -3) in the chicken oviduct regression and recrudescence. Cell Tissue Res. 366:443–454.

Leśniak-Walentyn, A., and A. Hrabia. 2017. Expression and localization of matrix metalloproteinases (MMP-2, -7, -9) and their tissue inhibitors (TIMP-2, -3) in the chicken oviduct during pause in laying induced by tamoxifen. Theriogenology. 88:50–60.

Li, S., Y. Wang, Y. Zhang, M. M. Lu, F. J. DeMayo, J. D. Dekker, P. W. Tucker, and E. E. Morrisey. 2012. Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2. Development. 139: 2500–2509.

Li, W., D. Sahu, and F. Tsen. 2012. Secreted heat shock protein-90 (Hsp90) in wound healing and cancer. Biochim. Biophys. Acta. 1823:730–741.

Makkar, S., R. Liyanage, L. Kannan, B. Packialakshmi, J. O. Lay, Jr, and N. C. Rath. 2015. Chicken egg shell membrane associated proteins and peptides. J. Agric. Food Chem. 63:9888–9898.

Millonig, G. 1964. Study on the factors which influence preservation of fine structure. Page 347 In: Symposium on Electron Microscopy, P. Buffa, ed. Consiglio Nazionale delle Ricerche, Rome.

Moll, R., W. W. Franke, and D. L. Schiller. 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell. 31:11–24.

Mork, A. K., M. Hesse, S. Abd El Rahman, S. Rautenschlein, G. Herrler, and C. Winter. 2014. Differences in the tissue tropism to chicken oviduct epithelial cells between avian coronavirus IBV strains QX and B1648 are not related to the sialic acid binding properties of their spike proteins. Vet. Res. 45:67.

Nio-Kobayashi, J. 2017. Tissue- and cell-specific localization of galectins,β-galactose-binding animal lectins, and their potential functions in health and disease. Anat. Sci. Int. 92:25–36.

Oze, H., M. Hirao, K. Ebina, K. Shi, Y. Kawato, S. Kaneshiro, H. Yoshikawa, and J. Hashimoto. 2012. Impact of medium volume and oxygen concentration in the incubator on pericellular

oxygen concentration and differentiation of murine chondrogenic cell culture. In Vitro Cell Dev. Biol. Anim. 48:123–130.

Palmiter, R. D., and J. T. Wrenn. 1971. Interaction of estrogen and progesterone in chick oviduct development. 3. Tubular gland cell cytodifferentiation. J. Cell Biol. 50:598–615.

Park, S. W., G. Zhen, C. Verhaeghe, Y. Nakagami, L. T. Nguyenvu, A. J. Barczak, N. Killeen, and D. J. Erle. 2009. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. Proc. Natl. Acad. Sci. USA. 106:6950–6955.

Pescatore, A. J., and B. J. Marquez 1977. Sperm storage tubule development in immature turkeys injected with female sex steroids. Poult. Sci. 56:1746–1747.

Pettersen, E. O., L. H. Larsen, N. B. Ramsing, and P. Ebbesen. 2005. Pericellular oxygen depletion during ordinary tissue culturing, measured with oxygen microsensors. Cell Prolif. 38:257–267.

Renoir, J. M., A. Pahl, U. Keller, and E. E. Baulieu. 1993. Immunological identification of a 50 kDa Mr FK506-binding immunophilin as a component of the non-DNA binding, hsp90 and hsp70 containing, heterooligomeric form of the chick oviduct progesterone receptor. C. R. Acad. Sci. III. 316:1410–1416.

Sanders, M. M., and G. S. McKnight. 1985. Chicken egg white genes: multihormonal regulation in a primary cell culture system. Endocrinology. 116:398–405.

Sasanami, T., M. Matsuzaki, S. Mizushima, and G. Hiyama. 2013. Sperm storage in the female reproductive tract in birds. J. Reprod. Dev. 59:334–338.

Schuppin, G. T., H. P. Van Krey, D. M. Denbow, M. R. Bakst, and G. B. Meyer. 1984. Ultrastructural analyses of uterovaginal sperm storage glands in fertile and infertile turkey breeder hens. Poult. Sci. 63:1872–1882.

Seaver, S. S., J. van der Bosch, and G. Sato. 1984. The chick oviduct in tissue culture. Exp. Cell. Res. 155:241–251.

Sever, D. M., and W. C. Hamlett. 2002. Female sperm storage in reptiles. J. Exp. Zool. 292:187–199.

Takeuchi, T., M. Suzuki, N. Fujikake, H. A. Popiel, H. Kikuchi, S. Futaki, K. Wada, and Y. Nagai. 2015. Intercellular chaperone transmission via exosomes contributes to maintenance of protein homeostasis at the organismal level. Proc. Natl. Acad. Sci. USA 112:E2497–E2506.

Talbot, N. C., L. A. Blomberg, W. M. Garrett, and T. J. Caperna. 2010a. Feeder-independent continuous culture of the PICM-19 pig liver stem cell line. In Vitro Cell Dev. Biol. Anim. 46:746–757.

Talbot, N. C., L. A. Blomberg, A. Mahmood, T. J. Caperna, and W. M. Garrett. 2007. Isolation and characterization of porcine visceral endoderm cell lines derived from in vivo 11-day blastocysts. In Vitro Cell Dev. Biol. Anim. 43:72–86.

Talbot, N. C., and W. M. Garrett. 2001. Ultrastructure of the embryonic stem cells of the 8-day pig blastocyst before and after in vitro manipulation: development of junctional apparatus and the lethal effects of PBS mediated cell-cell dissociation. Anat. Rec. 264:101–113.

Talbot, N. C., A. M. Powell, T. J. Caperna, and W. M. Garrett. 2010b. Proteomic analysis of the major cellular proteins of bovine trophectoderm cell lines derived from IVP, parthenogenetic and nuclear transfer embryos: reduced expression of annexins I and II in nuclear transfer-derived cell lines. Anim. Reprod. Sci. 120:187–202.

Talbot, N. C., W. O. Sparks, A. M. Powell, S. Kahl, and T. J. Caperna. 2012. Quantitative and semiquantitative immunoassay of growth factors and cytokines in the conditioned medium of STO and CF-1 mouse feeder cells. In Vitro Cell Dev. Biol. Anim. 48: 1–11.

Talevi, R., and R. Gualtieri. 2010. Molecules involved in sperm-oviduct adhesion and release.Theriogenology. 73:796–801.

Taylor, W. G., and R. F. Camalier. 1982. Modulation of epithelial cell proliferation in culture by dissolved oxygen. J. Cell. Physiol. 111:21–27.

Vanderlaag, K. E., S. Hudak, L. Bald, L. Fayadat-Dilman, M. Sathe, J. Grein, and M. J. Janatpour. 2010. Anterior gradient-2 plays a critical role in breast cancer cell growth and survival by modulating cyclin D1, estrogen receptor-α and survivin. Breast Cancer Res. 12:R32. doi: 10.1186/bcr2586.

Wang, P., Y. F. Wang, H. Wang, C. W. Wang, L. S. Zan, J. H. Hu, Q. W. Li, Y. H. Jia, and G. J. Ma. 2014. HSP90 expression correlation with the freezing resistance of bull sperm. Zygote. 22:239–245.

Watanabe, K., M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J. B. Takahashi, S. Nishikawa, S. Nishikawa, K. Muguruma, and Y. Sasai. 2007. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. 25:681–686.

Wodziak, D., A. Dong, M. F. Basin, and A. W. Lowe. 2016. Anterior gradient 2 (AGR2) induced epidermal growth factor receptor (EGFR) signaling is essential for murine pancreatitis-associated tissue regeneration. PLoS One. 11:e0164968. doi: 10.1371/journal.pone.0164968

Yang, K. T., C. Y. Lin, J. S. Liou, Y. H. Fan, S. H. Chiou, C. W. Huang, C. P. Wu, E. C. Lin, C. F. Chen, Y. P. Lee, W. C. Lee, S. T. Ding, W. T. Cheng, and M. C. Huang. 2007. Differentially expressed transcripts in shell glands from low and high egg production strains of chickens using cDNA microarrays. Anim. Reprod. Sci. 101:113–124.

Yoshimura, Y., K. Koike, and T. Okamoto. 2000. Immunolocalization of progesterone and estrogen receptors in the sperm storage tubules of laying and diethylstilbestrol-injected immature hens. Poult. Sci. 79:94–98.

Zhu, Q., H. B. Mangukiya, D. S. Mashausi, H. Guo, H. Negi, S. B. Merugu, Z. Wu, and D. Li. 2017. Anterior gradient 2 is induced in cutaneous wound and promotes wound healing through its adhesion domain. FEBS J. 284:2856–2869. doi: 10.1111/febs.14155.