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Efficient Source of Cells in Proximal Oviduct for Testing Non-Viral Expression Constructs in the Chicken Bioreactor Model and for Other *in Vitro* Studies*

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This work shows the usefulness of chicken oviduct epithelial cells (COEC) in evaluating the efficacy of non-viral expression vectors carrying human therapeutic genes. Secondly, an efficient source of progenitor COEC for *in vitro* studies is described. Within the distal part of the oviduct, weak to moderate expression of a trans membrane glycoprotein (CD44) was observed. Single cells presenting only weak expression of CD44 were found in magnum sections. *in vitro* cultured oviduct cells originating from the distal oviduct were suitable for subculturing and showed a stable proliferation potential up to the 2nd passage. However, the pavimentous epithelial-like morphology of COEC was progressively lost over time and mainly a fibroblast-like monolayer was established in consecutive passages. Moreover, various commercial transfection agents including FuGENE6 and XtremeGENE9 DNA were used to optimize delivery of human interferon alfa-2a, (IFNα2a) a therapeutic protein gene under an ovalbumin promoter. The transfection efficiency of adherent COEC was estimated for up to 40% at a ratio of 6:1 of transfectant to pOVA5EIFN + GFP plasmid. Expression of IFNα2a was confirmed by western blotting in transformed COEC. In conclusion, the population of epithelial progenitor cells sourced from the distal oviduct can significantly contribute to *in vitro* culture of COEC, representing an efficient model to develop the production of avian bioreactors and other *in vitro* studies related to oviduct tissue.

Key words: Chicken oviduct, oviduct epithelial cell culture, COEC, avian bioreactor, CD44, non-viral transfection, distal magnum.

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Avian oviduct epithelial cells serve as a good model for studying the expression of exogenes delivered by DNA carriers in order to induce the production of human therapeutic proteins in egg white of avian bioreactors (LEE *et al*. 2013; CHOJNACKA-PUCHTA *et al*. 2012; JUNG *et al*. 2011; KAMIHIRA *et al.* 2009). Some results might be extrapolated to human studies related to the hormonal regulation of reproductive tract and diseases caused by pathogens such as avian influenza virus or *Salmonella*. Many authors find clear indications that the oviduct of a laying hen might resemble malignancies occurring in the female reproductive tract. Thus, an oviduct model is highly applicable in studies on the genesis of a serous type of ovarian cancer in women (JOHNSON 2014), research on cancer stem cell markers (PAIK *et al*. 2012) and development of tools for its diagnosis or treatment (BARUA *et al*. 2015; LIM *et al*. 2015). However, certain issues need to be resolved to increase the effectiveness and quality of such studies. None of currently proposed viral transgenic systems is ready for routine implementation in a bioreactor approach. The main reason is low efficiency of the genetic constructs, not to mention restrictions related to biosecurity when using viral vectors (PARK *et al*. 2015; SAWICKA *et al*. 2015). Slow progress is also connected with a deficit of avian antibodies and tools for *in vitro* testing of avian biological material.

Therefore, different methodologies to transfect chicken oviduct epithelial cells (COEC) in a non-viral way are proposed in this work. Also, the acute source of proliferating COEC suitable for an *in vitro* model is determined using appropriate markers. The expression of the CD44 cell adhesion molecule in distal sections of hen oviduct tissue is demonstrated for the first time. A key advantage of the proposed *in vitro* model is a simple and fast protocol (4-7 days) to assess effectiveness of different exogenous carriers driving expression of recombinant therapeutic proteins in hens. We demonstrate the feasibility of chicken oviduct cells to verify effectiveness of several non-viral transfection agents including DDAB/DOPE, FuGENE 6 Transfection Reagent, Nanofectamin and XtremeGENE 9 DNA Transfection Reagent as carriers of different variants of human interferon alfa-2a (IFNα2a) expression vector. Our results might also provoke a discussion as to whether translating the chicken model to humans is rational for studying disorders of the reproductive tract.

Material and Methods

Abbreviations used:

COEC – chicken oviduct epithelial cells,
DDAB/DOPE – dimethyldioctadecylammonium bromide/dioleylphosphatidylethanolamine,
PBS – physiological buffered saline,
DMEM/F-12 – Dulbecco's modified Eagle's medium,
FBS – fetal bovine serum/,
GFP – green fluorescent protein,
RT-qPCR – reverse transcription quantitative PCR,
DAB – 3-3'diaminobenzidine

Establishment of COEC cultures

Oviduct tissues used for experiments were dissected from randomly selected, 30 to 40-wk-old commercial lying hens: Tetra SL and Hy-line Brown hybrids (n=10), after egg massage passage. The birds were obtained from a certified farm in Prądocin 24, 86-060, Poland, sacrificed according to the regulations of Polish Local Ethical Commission, permission no. 35/2012 from 13.07.2012 and Directive 2010_63_UE_PL.

Cell cultures of COEC were carried out using the protocol of KASPERCZYK *et al*. (2012). Briefly, a distal section of oviduct tissue, 5 cm long, was trimmed of mesentery and superficial connective tissue with forceps and microscissors and washed several times in PBS with addition of 10% (v/v) penicillin/streptomycin solution (Cat. 03-031-1B Biological Industries, Genos, Poland) and amphotericin B (Cat. 03-028-1B Biological Industries, Genos, Poland). Dissected tissue was finely minced and digested with 1 mg/ml collagenase P (Cat. 11213865001 Roche, Poland) in Advanced DMEM/F-12 (Cat. 12634-010 Life Technologies, Poland) on a rotary shaker for 30 min at 37°C. Immediately after digestion, the samples with dissociated oviduct fragments were centrifuged 3 times at 760 × g for 5 min and washed twice with DMEM/F-12 containing 10% (v/v) FBS (Cat. 16140-063 Life Technologies, Poland). Prior to final centrifugation, a turbid suspension of oviduct tissue fragments was filtered through 100 µm nylon mesh (Cat. 352360 BD Falcon, Diag-Med, Poland). The cells were then dispersed in 2 ml of culture medium DMEM/F-12 with 10% FBS and their number was estimated with a Neubauer improved chamber. Seeding density was 1 x 10^6 cells/ per well in 12-well culture plates (Cat. 353043 Corning Life Sciences, Poland). Upon reaching over 70% of growth confluence, the cells were passaged into 25 cm^2 bottles using Reagent Pack Subculture Reagents (Cat. CC-5034 Lonza, Celllab, Poland). Evaluation records were prepared by two independent observers with two visualizing systems: an inverted microscope (Axiovert 40, Zeiss) and JuLI Smart Fluorescent Analyser (ALAB, Poland).

Transfection of COEC

Two consecutive transfection trials T1 and T2 were designed, firstly to test the efficiency of 4 dif-

ferent transfection agents (T1) and secondly to test the efficiency of 3 non-viral expression vectors containing the human IFNα2a gene (T2) (Bioengineering Department, Institute of Biotechnology and Antibiotics, Warsaw).

Experiment T1 to test the efficiency of transfection agents. Trial T1 was carried out using pOV5EINT + GFP (9245bp) expression construct containing green fluorescent protein (GFP) as a reporter gene, inserted next to the human IFNα2a gene under control of the OVA promoter with enhancer sequences and Estrogen Responsive enhancer Element – ERE, (GeneBank, cat. S82572S1) (LILLICO *et al*. 2007; KATO *et al*. 1992) at the 5' end. The pOVA5EIFN + GFP expression construct was diluted at a concentration of 0.5 μg/1 μl in TE buffer. All 4 transfection agents, each in 3 repetitions, were tested in order to select the most effective one for adherent cells: DDAB/DOPE (courtesy of Aleksander Sochanik, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland), FuGENE6 Transfection Reagent (Cat. E2691 Promega, Poland), Nanofectamin (Cat.Q0002-006 PAA, GE Healthcare Immuniq, Poland) or XtremeGENE9DNA Transfection Reagent (Cat. XTG9-RO Roche, Sigma-Aldrich, Poland). Post transfection analysis was conducted by microscopic evaluation of GFP expression using an inverted microscope (Axiovert 40, Zeiss) with 450-490 nm filter and a confocal microscope (Nikon Eclipse) with 360-460 nm filter for DAPI counterstain.

Transfection of COEC in suspension with DDAB/DOPE. In order to transfect COEC, electroporation was combined with lipofection. Cationic lipids DDAB and auxiliary liposomes DOPE were used, according to the method of WAWRZYŃSKA *et al*. (2008). A negative control was established from undifferentiated chicken blastoderm cells (BCs) transfected with pOVA5EIFN + GFP construct using the method of BEDNARCZYK *et al*. (2003). The liposomic solution (LS) consisted of 16 μl DDAB/DOPE stock fivefold diluted in 64 μl 150 mM NaCl. A single electroporation solution (ES) consisted of 5 μl of plasmid diluted in 500 μl of isoosmotic buffer (Cat. 4308070.510 Eppendorf, Meranco, Poland). A single transfection solution (TS) was prepared by instilling 5 μl of diluted expression vector into 66.4 μl of OptiMEM, Reduced Serum Medium (Cat.11058-021 Life Technologies, Poland). To perform single transfection, the LS was combined with 320 μl of OptiMEM, incubated for 15 min at RT, added to 80 μl TS and incubated for a further 15 min. COEC were detached from cultureware using Trypsin-EDTA (Cat.T4049 Sigma-Aldrich, Poland) suspended in 4.5 ml OptiMEM, centrifuged for 15 min at 670 rcf and mixed with earlier prepared ES. Such prepared cells were placed immediately into an electroporation cuvette in a Multipolator device (Cat. 4308000.015, Eppendorf, Poland) and transfected with 3 electric pulses at 25 V, at 500 μs pulse duration moment, with 1 min intervals between the pulses. Directly after electroporation, the cells were removed and centrifuged for 5 min at 670 rcf. The pellet with COEC was suspended again in 200 μl of OpitMEM and mixed with LS. Lipofection lasted 4 h, at 37°C in 5% CO_2. Then the cells were centrifuged for 10 min at RT, suspended in cultivation medium and cultured for a further 48 h until GFP expression analysis.

Transfection of the adherent COEC with FuGENE6. The COEC were cultured in 12-well culture plates until reaching 70% growth confluence. The growth medium was exchanged 1 day prior to transient transfection. Two different ratios of FuGENE6 to plasmid pOVA5EIFN + GFP (3:1, 6:1) were tested. A single transfection solution of 50 μl was prepared according to the manufacturer's protocol and added dropwise directly to the cultured COEC. The cells were incubated for a further 48 h until GFP expression analysis.

Transfection of the COEC by Nanofectamin. COEC were cultured in 12-well plates until reaching 70% growth confluence. Growth medium was exchanged 1 day before the scheduled transfection. Two different ratios of Nanofectamin to plasmid pOVA5EIFN + GFP were tested (2:1, 6:1). A single transfection solution of 100 μl was prepared according to the manufacturer's protocol and added dropwise directly to the cultured COEC. After 4 h incubation, the transfection solution was changed to growth medium and COEC were cultured for a further 48 h until GFP expression analysis.

Transfection of the COEC by XtremeGENE9DNA. Two different ratios of XtremeGENE9DNA Transfection Reagent to plasmid pOVA5EIFN + GFP were tested (2:1, 6:1). A single transfection solution of 100 μl was prepared according to the manufacturer's protocol and added dropwise directly to the cultured COEC. After transfection, the cells were incubated for a further 48 h until GFP transient expression analysis.

Experiment T2 to test the efficiency of different plasmids expressing IFNα2a. The second experiment, T2, was carried out based on the optimisation of results of trial T1. XtremeGENE9DNA was appointed for the best performing transfection agent and for this reason it was used in Trial 2. The objective of the T2 experiment was to evaluate the efficiency of 3 different expression plasmids which deliver the human IFNα2a gene specifically into COEC, which was confirmed by Western

blotting. Key elements of each tested construct were as follows:
1) in pOVAINTEnh (6852 bp): chicken ovalbumin promoter (OVA) (2913 bp, GeneBank No. J00895), human IFNα2a gene and SV40 enhancer sequence (from pGL3-Control, Promega).
2) in pOV5INT (6803 bp): human IFNα2a gene under control of the OVA promoter with enhancer sequences – COSE element (PARK *et al*. 2006) at the 5' end (~ 1000 bp).
3) in pOV5EINT (7472 bp): human IFNα2a gene under control of the OVA promoter with enhancer sequences and the Estrogen Responsive enhancer Element – ERE, (GeneBank, cat. S82572S1) (LILLICO *et al*. 2007; KATO *et al*. 1992) at the 5' end.

Western blotting analysis of IFNα2a in COEC lysates

Culture wells with transfected cells were rinsed 3 times with PBS (pH 7.4) and filled with 300 µl of extraction buffer containing 5% (v/v) 1 M TRIS (pH 7.5), 5.36% (v/v) 2M NaCl, 1% (v/v) TritonX-100, 10% (v/v) glycerol and of 15 µl/ml Protease Inhibitor Cocktail (Cat.P8340, Sigma-Aldrich, Poland). The cells, kept on dry ice, were quickly extracted from culture wells using cell scrapers, transferred to 1.5 ml tubes and centrifuged for 15 min at 4°C. Supernatant was deep frozen and stored for no more than 2 wks. Immediately prior to analysis, the COEC extract was concentrated with Millipore Amicon Ultra-0.5ml 10 K columns (Cat.UFC501024, Merck Millipore, Poland). The minimal concentration of protein sample was determined based on a chemiluminescence assay with the IFNα2a 19.8 kDa protein standard (Cat.NBC1-21338, Novus Biologicals, Prospecta, Poland). Total proteins in COEC samples were determined against the Bradford standard curve on a UV-VIS Evolution 60 spectrophotometer (Thermo Scientific) at 595 nm. Samples containing at least 5 µg proteins in 25 µl were subjected to SDS-PAGE electrophoresis, transferred to a PVDF membrane with a Trans-Blot®SD SemiDry Electrophoretic Transfer Cell device (Cat.170-3940, BioRad, Poland) and incubated with MaxPab mouse polyclonal antibody H00003440 IFNA2 Pab (Cat.B01P, Abnova, Cytogen, Poland) at 1500 dilution at 4°C o/n, washed 8 times for 8 min with a buffer containing 50% (v/v) 1 M TRIS pH 7.5, 30% (v/v) 5 M NaCl, 0.5% (v/v) 0.2 M EDTA pH 8.0 and 1% (w/v) Tween 20, followed by incubation with a goat polyclonal secondary antibody to mouse IgG-H&L horseradish peroxidase (HRP) (Cat.ab97023, Abnova, Cytogen, Poland) at 20000 dilution for 1h at RT followed by 8 washing steps with the same PVDF membrane washing buffer. The chemiluminescent reaction was performed with ECL Plus Western Blotting Detection Reagent (Cat.RPN2132, Amersham GE Healthcare, GE Medical Systems Polska Sp.z o.o.) by 5 min incubation on Kodak Biomax Light (Cat.Z373508-50EA, Sigma Aldrich, Poland).

Preparation of tissue samples for immunohistochemical analysis (IHC) with CD44 antibody

Oviduct fragments, each 4 cm long, were dissected from the infundibulum (INF) distal magnum (DM) and proximal magnum (PM). IHC was performed on paraffin embedded formalin fixed tissue sections, 8 sections per each INF, DM and PM fragment. Tissue sections were fixed in 10% buffered formalin and processed according to a standard protocol (dehydrated in ethyl alcohol 80%-99.8%), cleared in xylene (I-IV), and embedded in paraffin. CD44 is considered a marker of basal epithelial layers of cells differentiating into epithelial cells and a marker of epithelial- mesenchymal transition states. IHC staining of tissue sections was performed using primary mouse monoclonal antibody CD44 (Clone DF1485 Dako, Glostrup, Denmark). Epitopes were unmasked using Epitope Retrieval Solution high-pH (Dako, Glostrup, Denmark). The endogenous activity of peroxidase was blocked using 3% H_2O_2 for 15 min, and non-specific binding was blocked using 5% bovine serum albumin (BSA, Sigma-Aldrich, Poland) in a phosphate buffered saline (PBS) for 10 min at RT. Subsequently, the slides were incubated with the primary antibody at 4°C o/n (dilution 1:25). Antibody complex was detected using EnVision Flex Anti-Mouse/Rabbit HRP-Labeled Polymer (Dako, Glostrup, Denmark). Antigens were localized using DAB as chromogen. The sections were counterstained with haematoxylin, dehydrated in increasing grades of ethyl alcohol, cleared in xylene and mounted. Results were analysed using a light microscope ECLIPSE E400 (Nikon Instruments Europe, Amsterdam, Netherlands) at original objective magnification: 20X. Analysis of protein expression was performed using a modified Remmele-Stegner scale (Index Remmele-Stegner IRS 0-3) based on intensity of positively stained cells as the following: 0 – no expression, 1 – weak expression, 2 – moderate expression, 3 – strong expression.

RNA extraction and RT-qPCR

In the RNA isolation step, 4 cm long oviduct fragments, were homogenized in TRI reagent (MRC, Cincinnati, USA) using TissueRuptor

(Qiagen, Venlo, Netherlands) and further purified (Universal RNA Purification Kit, EURx, Gdansk, Poland). Total RNA was controlled by using a spectrophotometer and agarose gel electrophoresis. cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania) according to the manufacturer's recommendations. Prior to qPCR, amplification obtained cDNA was diluted to a working concentration of 70 ng/µl. RT-qPCR was performed by a LightCycler 480 Instrument II (Roche Diagnostics, Basel, Switzerland). Reactions were conducted in a total volume of 10 µl which included Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific/Fermentas, Vilnius, Lithuania), 1 µM each of primers: forward 5'-ACGAGGAGCAAAGCATGTGA-3' and reverse 5'-GTGAGCCGTCCTCATTGTCA-3', 1 µl of cDNA. Primers were designed using NCBI Primer – BLAST based on the CD44 sequence (accession: NM_204860.2). Primers spanned an exon-exon junction to avoid gDNA amplification. The qPCR thermal program consisted of 15 min initial denaturation at 95°C and 40 cycles of amplification: 10 s denaturation at 95°C, 15 s primer annealing at 58°C, 30 s primer extension at 72°C. A melting curve to assess the efficiency of the RT-qPCR reactions with a gradual temperature increase up to 98°C and a continuous measurement of fluorescence was further generated. Relative quantification analysis was performed by using the ddCt method (LIVAK & SCHMITTGEN 2001) and ubiquitin C as a reference gene (DE BOEVER *et al.* 2008).

Results

The avian oviduct cell cultures

Over 70% of the total cell population isolated from the distal part of the oviduct developed colonies *in vitro*, but only 50% retained proliferation capacity which was highest at passage 2. After the 2nd passage the cells became senescent. The highest capacity of cells to adhere to a vessel surface and proliferate after passaging was observed in samples obtained from the most distal fragments of the oviduct (infudibulum neck/ upper -distal magnum). The process of adhesion lasted from 3 to 7 days and cultured cells displayed a typical epithelial colony morphology, which would likely develop into a heterogenous fibroblast or mesenchymal-like spindle shaped (SS) and epithelial-like rectangular shape (RS) monolayer (Fig.1).

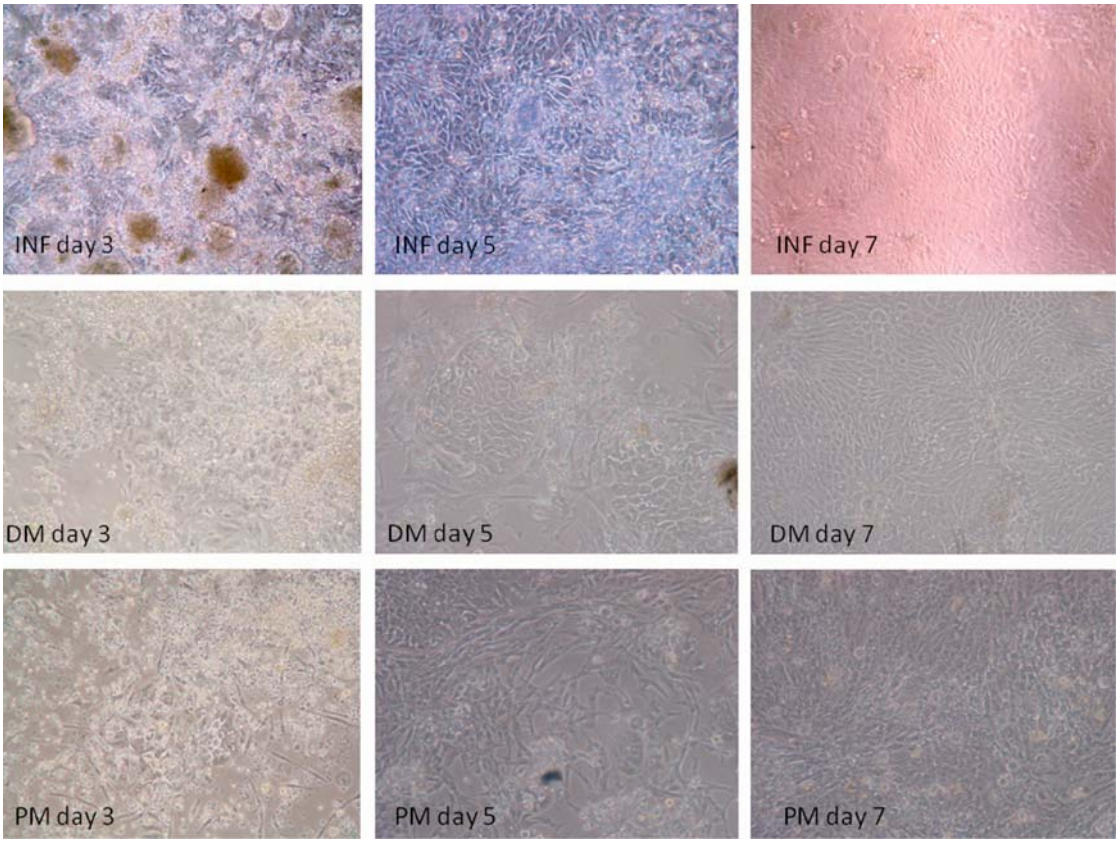

Fig. 1. Development of chicken epithelial colony cells. Representative populations were isolated from 3 distal parts of oviduct: infundibulum (INF), distal magnum (DM) and proximal magnum (PM). Epithelial colony cells were sub-cultured onto 12-well plates; on day 5-7 the cells were examined and transfected with expression constructs: pOVAINTEnh, pOV5INT and pOV5EINT.

Transfection of COEC with various transfection agents

Only low, 30% recovery of COEC following electroporation and lipofection with DDAB/DOPE was detected. Following transfection with FuGENE6, COEC survivability was 90% and a strong fluorescence of COEC aggregates was observed 48h post transfection in a maximum of 40% of the cell population. The level of IFNα2a exogene incorporation increased with higher FuGENE6: plasmid pOVA5EIFN + GFP ratios and was optimal at a 6:1 ratio (Fig. 2). Following transfection with Nanofectamin, 80% a survivability rate was reported and 20% of the population expressed GFP regardless of the Nanofectamin: plasmid pOVA5EIFN + GFP ratios applied (Fig. 3 A-B). A high survivability rate (90%) and the highest transfection efficiency (40%) was found in COEC transfected with XtremeGENE9DNA agent at a 6:1 ratio (Fig. 3 C-D). The latter method, due to its low cytotoxicity and the highest transfection efficiency, was recommended to conduct experiment T2. Western blotting analysis showed the expression of IFNα2a corresponding to the 19.8 kDa band (Fig. 4).

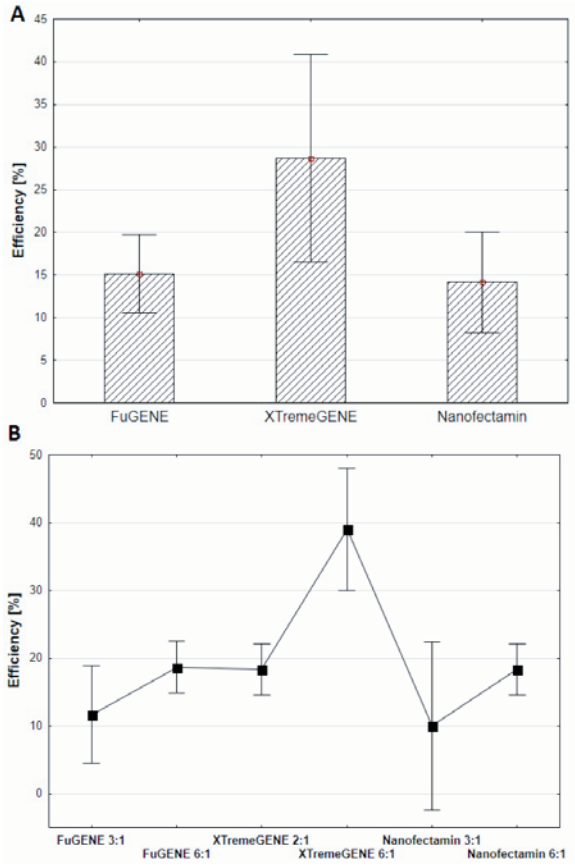

Fig. 2. Optimization of transfection conditions. Ratio of FuGENE6 transfection reagent to plasmid pOVA5EIFN + GFP as optimized to deliver IFN α2a gene into chicken oviduct epithelial cells. Each experiment was performed at least in triplicate. A – represents the amount of cells expressing GFP with three transfectant reagents: FuGENE6, Nanofectamin and XtremeGENE (P>0.05). B – illustrates the amount [%] of cells expressing GFP after applying different ratios of transfectant reagent to plasmid pOVA5EIFN + GFP plasmid (P=0.05). The obtained data is presented as means ± standard deviation. Statistical analysis was performed by nonparametric comparisons of groups with χ^2 test (STATISTICA v.10).

Expression of CD44 in the hen oviduct

Various expression levels of CD44 were shown in 8 histological sections in the oviduct infundibulum. A low expression level was found in sections no. 1-3 of the distal infundibulum (Fig. 5 A), moderate expression of CD44 was found in sections no. 4-6 of the middle infundibulum (Fig. 5 C-INF), and low expression of CD44 was found in section no. 7 of the proximal infundibulum whilst no expression was observed in section no. 8 (Fig. 5 A). In the sections of magnum, only single cells showed expression of CD44 in both distal and proximal areas (Fig. 5 C-DM, C-PM). RT-qPCR confirmed these results and the highest level of CD44 expression was found in the distal oviduct (2.5 times higher fold induction vs. control tissue), whilst a gradually decreasing expression of CD44 was found in the proximal parts of the magnum (Fig. 5 B).

Discussion

Ovulatory cycles are bound to dynamic remodeling of tissue involving the activity of stem cells. INDUMATHI *et al*. (2013) found that stem cells from woman endometrium and fallopian tube express markers of pluripotency and a range of mesenchymal stem cell markers including SSEA4, OCT4, SOX2 and cell adhesion molecule CD44. The latter is expressed in normal and cancer stem cells in the fallopian tube (PAIK *et al*. 2012). LIM & SONG (2013) support the hypothesis that oviduct regulatory genes, e.g. SERPINB3, SERPINB11 or SPP1 are candidates for critical regulators of ovarian carcinogenesis in laying hens and might play a role in the diagnosis of human epithelial derived ovarian cancer. The avian urogenital tract is considered one of the most carcinogenesis prone systems among vertebrates and neoplasms in domestic poultry might be divided into two main types depending on their origin: tumors caused by viruses e.g. Marek's disease virus, reticuloendotheliosis, avian leukemia and tumors of unknown etiology. Some authors indicate that cell cultures from malignant birds may significantly expand prospects in viral oncology and chemoprevention trials (HALES *et al*. 2014; MOTIANI *et al*. 2013). The pursuit of stem cell markers in the fallopian tube has been reflected in recent publications. In mice, results obtained by SNEGOVSKIKH *et al*. (2014) showed that putative stem cells are located

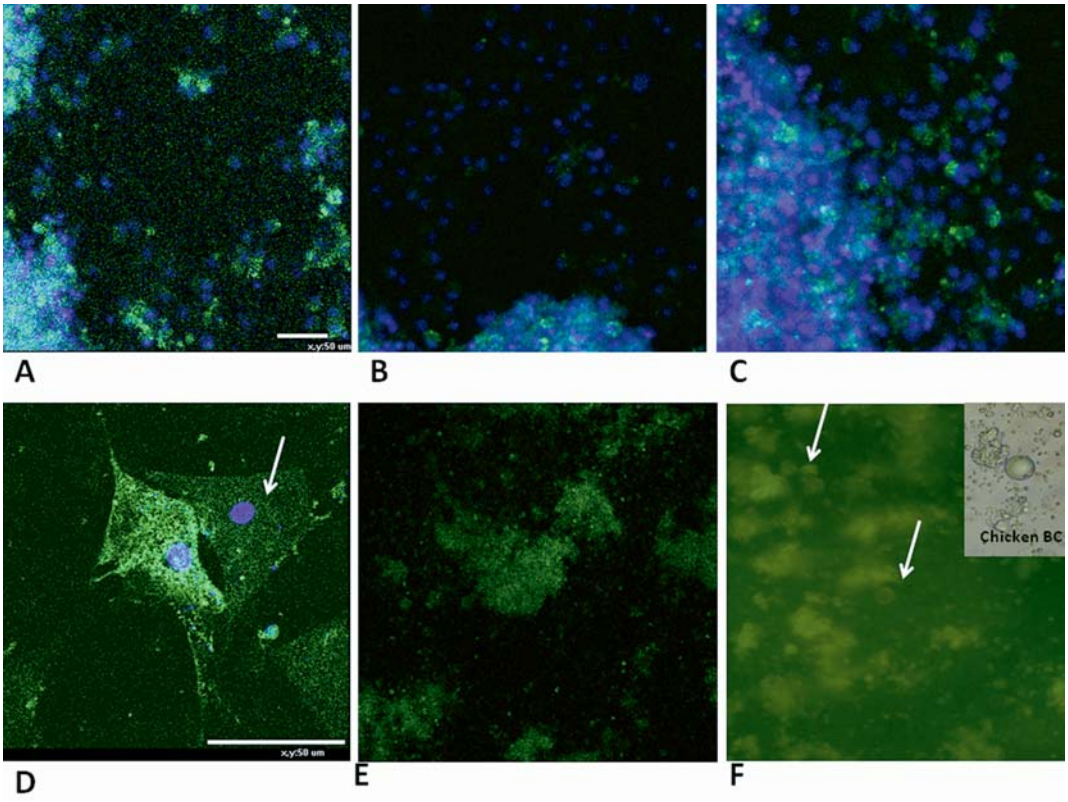

Fig. 3. Expression of exogenes delivered into chicken oviduct epithelial cells with non-viral expression system. GFP -IFN α2a expression construct was delivered with Nanofectamine at two different ratios of transfectant to DNA: ratios 3:1 (A) and 6:1 (B), and with X-tremeGENE at transfectant to DNA ratio 6:1 (C-D). Two types of control samples were established: E- non transfected COEC observed with two types of fluorescent microscopes (Nicon Exlipse, Zeiss Axiovert,) in order to eliminate the autofluorescence errror, and F- chicken blastodermal cell (BCs), not expressing ovalbumin (OVA), thus not responding to genetic constructs under the OVA promoter. DAPI staining was performed to display all cell nuclei at magnification 20x (A-C) and 100x (D). The white bands represent 50 µm. Arrows indicate non-transfected cells.

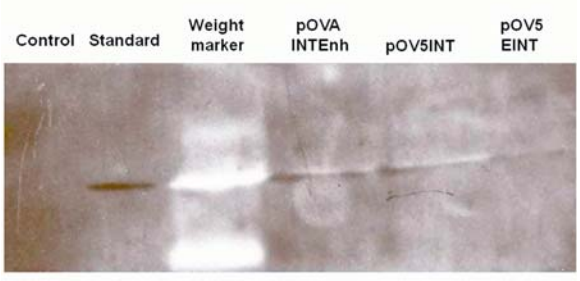

Fig. 4. Western blotting bands representing IFN α2a detected in modified chicken oviduct epithelial cells from the *in vitro* culture. Names pOVAINTEnh, pOV5INT and pOV5EINT, represent the applied expression constructs.

at the base of each villi in the fallopian tube, suggesting the location of a stem cell niche accounting for 0.5% of the cell population. CAPEL (2014) appointed Lgr5 as a potential specific marker of cells driving the regeneration of ovarian epithelium. In parallel, another group used the same marker to identify progenitor cells in ovary and tubal epithelia (NG *et al*. 2014). As previously reported by TREVIÑO *et al*. (2010) for stem like cells or STADNICKA *et al*. (2014) for epithelial progenitor cells, their niche is most likely located in the distal oviduct close to the ovaries. PAIK *et al*. (2012) attempted to characterize epithelial cells in human fallopian tube and found that undifferentiated cells localized in the basal epithelium in a distal oviduct section expressed surface antigens CD44 and integrin á6. These cells lacked markers of ciliated or secreting epithelial cells. This finding is in agreement with our observation as we show the expression of CD44 on epithelial cells located basally in the distal oviduct of a lying hen. This is confirmed by both RT-qPCR (Fig. 5 B) and IHC (Fig 5 C), as well as by a high proliferative potential and good *in vitro* development of cells sourced from distal oviduct tissue (Fig. 1). Chicken cells isolated from distal oviduct and grown *in vitro* retain proliferative potential up to several passages whereas epithelial cells isolated from proximal sections of oviduct magnum do not retain the potential to proliferate after passaging and display early senescence. *in vitro*, suspensions of cells isolated from the distal oviduct are typically rich in yellowish-gold spherical multi-cellular aggregates accounting for several tens to several hundred cells. These cell aggregates adhere to the culture plate surface and initiate fast proliferating epithelial-like colonies after 2-3 days of culture (Fig. 1). These cells express epithelial progenitor cell marker p63 as previously reported (STADNICKA *et al*. 2014;

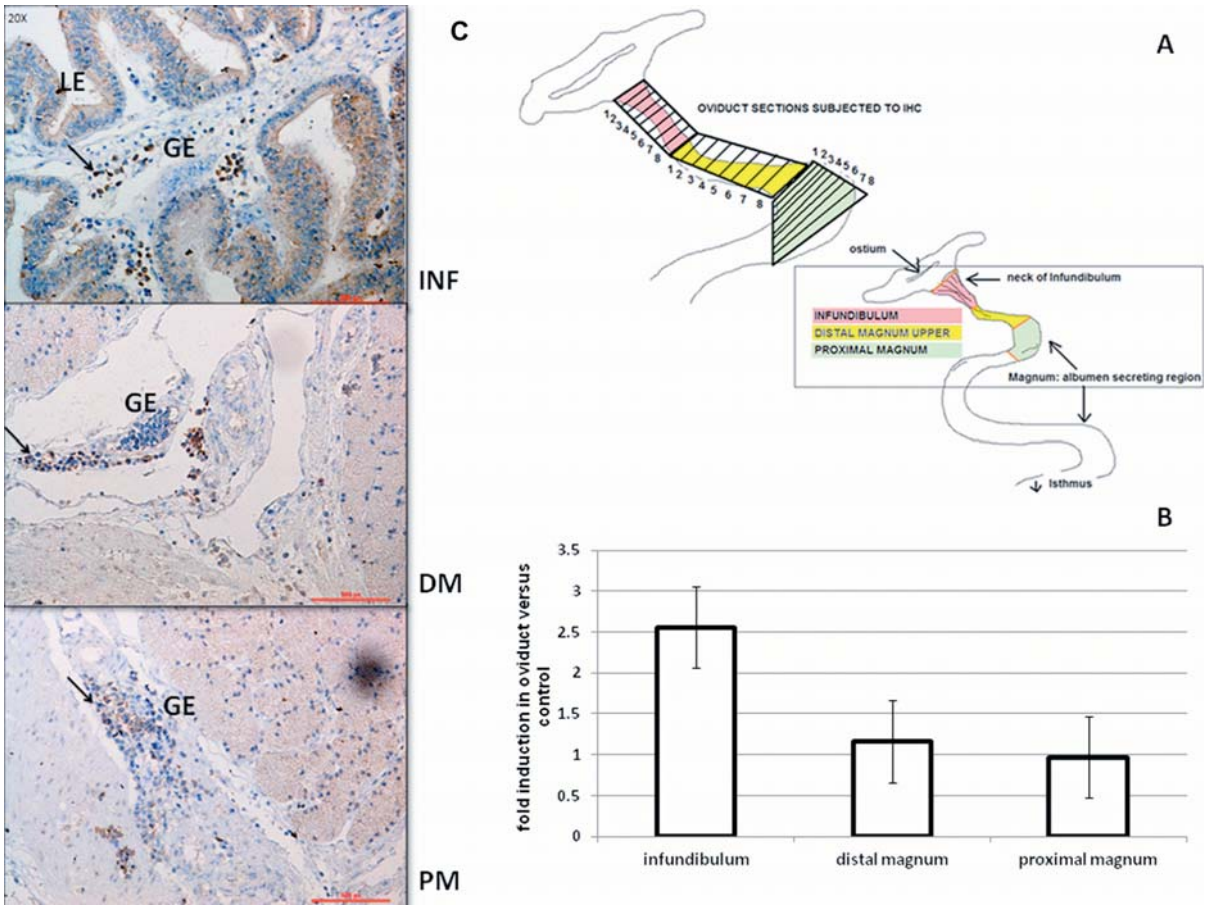

Fig. 5. CD44 stain and CD44 mRNA expression in the oviduct of a laying hen (age 33-wks). Panel A – scheme of sampling for immunohistochemistry (IHC). Three parts of oviduct: INF – infundibulum, DM – distal magnum and PM – proximal magnum were divided into 8 histological sections each. IHC stain of a single section was considered as one repetition. Three replicates were done for each section. Panel B – graph showing fold change in mRNA expression of the CD44 gene in oviduct fragments versus control (pectoralis major muscle tissue) quantified by RT-qPCR. Panel C – Representative images of CD44 stain in three oviduct fragments: INF, DM and PM. LE = luminal epithelium, GE = glandular epithelium.

KASPERCZYK *et al*. 2012). Earlier, PALMITER & WRENN (1971) stated that the transition of oviduct cells from the proto-differentiated stage into secreting type might be subordinated to regulation of estrogen and progesterone. Recently KHUONG & JEONG (2011) showed adipogenic potential of COEC after treatment with chicken serum *in vitro*. However, we do not confirm this finding. In our study, neither the adipogenic, chondroigenic nor osteogenic potentials of hen oviduct cells were induced by differentiation media (personal communication). In hen, cells located basally in oviduct epithelium crypts should be considered uni-potent progenitor epithelial cells in contrast to $CD44^{+}$ cells of the mammalian fallopian tube which are claimed to represent so called peg-cells or the stem like cell population (GARSON & VANDERHYDEN 2015; PAIK *et al*. 2012). In contrast to the findings regarding female fallopian tube, we have not confirmed the mesenchymal or multipotent character of cultured chicken cells obtained from the distal oviduct.

Our results show the usefulness of the *in vitro* COEC model for studying the efficiency of transfection tools and the development of avian bioreactors. The secreting potential of cells from the oviduct infundibulum is often disregarded, but we found that after transformation with expression plasmid under the ovalbumin promoter containing exogenous FN-β1 or IFNα2a sequence, these cells produced a nonnative protein *in vitro* (CHOJNACKA-PUCHTA *et al*. 2015). Some cells isolated from infundibular epithelium might exhibit similar features as tubular gland cells, perhaps due to the rapid differentiation of their precursors *in vitro*. In favor of avian bioreactor research, the post transcriptional pattern for glycosylation of proteins in avian species is consistent with that occurring in human, e.g., transgenic quail produced hIL1RN containing a normal N-glycosylation site with bioactivity similar to the commercially available ver-

sion of a bio-functional therapeutic protein known as anakinra (KWON *et al*. 2010). We recommend modifying adherent COEC with high rations of commercially available transfection agents, in particular FuGENE6 Transfection Reagent or XtremeGENE9DNA Transfection Reagent. The most forceful DNA expression vectors in avian studies are composed of viral elements (LEE *et al*. 2013). Nevertheless, their application is challenging owing to a number of controversies related with potential side effects such as insertional mutagenesis, inhibition of cellular genes (ISHII & MIKAWA 2005), transactivation of endogenous genes and positional effects (SCOTT & LOIS 2005). Likewise the mere fact of using lentiviruses, which are recognized as malicious pathogenic factors, puts the future of viral vectors in bioreactor applications into question. In this work, high expression efficiency of GFP (40%) was observed after non-viral transformation of COEC (Fig. 3). The inserted IFNα2a gene was identified in transformed COEC with a PCR protocol according to SIWEK *et al*. (2010) and by Western blotting (Fig.4). Earlier, CHOJNACKA-PUCHTA and colleagues (2012) showed that up to 30 ng of IFNβ1 was expressed in COEC *in vitro* by the same method. Several authors recognized that FuGENE HD (Roche Diagnostics), an updated version of FuGENE6, was the most effective non-viral transfection agent for many cell lines including those of epithelial character (YAMANO *et al*. 2010; DICKENS *et al*. 2010). In contrast to other liposomic agents, transfection with FuGENE6 can be performed in the presence of serum which obviously facilitates *ex vivo* gene delivery protocols. X-TremeGene9DNA from Roche Diagnostics is a non-liposomal reagent which requires only minimal optimization procedures, hence both mentioned reagents may be used interchangeably depending on the requirements of cell culture conditions. This study shows that transfection efficiency of Nanofectamin was 3 times lower compared to the manual given by the manufacturer, nevertheless amendments to the protocol in terms of transfectant to plasmid DNA ratios might improve the outcome up to 60%, which was shown by BIAŁAS *et al*. (2011) in mouse myoblasts. Here, the observed expression level of GFP in COEC (20-40%) can be considered reasonably high if one takes into account the sensitivity of epithelial cells in primary cultures. Whereas in pure cell lines, like CHO, the efficiency of such transformation is usually expected at a minimum rate of 40-50%. Epithelial cells show susceptibility to potential toxicity of reagents, easily detach from culture vessels, and might degenerate in a short time after transfection. Moreover, the transfectant agent itself might promote cell differentiation, e.g. into fibroblast-like cells.

Summarizing, the hen oviduct is good and easily accessible material to study important biological mechanisms *in vitro* as well as to develop scalable research to deliver exogenes for human treatment by testing various expression constructs *in vitro*/*ex vivo* prior to in field testing.

References

BARUA, A., Yellapa A., Bahr J.M., Adur M.K., Utterback C.W., Bitterman P., Basu S., Sharma S., Abramowicz J.S. 2015. Interleukin 16- (IL-16-) Targeted Ultrasound Imaging Agent Improves Detection of Ovarian Tumors in Laying Hens, a Preclinical Model of Spontaneous Ovarian Cancer. Biomed. Res. Int. 2015: Article ID 567459, 10 pages.

BEDNARCZYK M., PŁUCIENNICZAK G., PŁUCIENNICZAK A., ŁAKOTA P., SOCHANIK A., DLUŻNIEWSKA P., GRAJEWSKI B. 2003. Expression of exogenous genes in blastodermal cells of chicken *in vitro*. Folia Biol. (Kraków) **51**: 189-194.

BIAŁAS M., KRUPKA M., JANECZEK A., ROZWADOWSKA N., FRACZEK M., KOTLINOWSKI J., KUCHARZEWSKA P., LACKOWSKA B., KURPISZ M. 2011. Transient and stable transfections of mouse myoblasts with genes coding for pro-angiogenic factors. J. Physiol. Pharmacol. **62**: 219-228.

CAPEL B. 2014. Ovarian epithelium regeneration by Lgr5(+) cells. Nat. Cell Biol. **16**: 743-744.

CHOJNACKA-PUCHTA, L., SAWICKA D., ŁAKOTA P., PŁUCIENNICZAK G., BEDNARCZYK M., PŁUCIENNICZAK A. 2015. Obtaining chicken primordial germ cells used for gene transfer: *in vitro* and *in vivo* results. J. Appl. Genet. **56**: 493-504.

CHOJNACKA-PUCHTA L., KASPERCZYK K., PŁUCIENNICZAK G., SAWICKA D., BEDNARCZYK M. 2012. Primordial germ cells (PGCs) as a tool for creating transgenic chickens. Pol. J.Vet. Sci **15**: 181-188.

DE BOEVER S., VANGESTEL C., DE BACKER P., CROUBELS S., SYS S.U. 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. Vet. Immunol. Immunopathol. **122**: 312-317.

DICKENS S., VAN DEN BERGE S., HENDRICKX B., VERDONCK K., LUTTUN A., VRANCKX J.J. 2010. Nonviral transfection strategies for keratinocytes, fibroblasts, and endothelial progenitor cells for *ex vivo* gene transfer to skin wounds. Tissue Eng. Part C Methods **16**: 1601-1608.

GARSON K., VANDERHYDEN, B. 2015. Epithelial ovarian cancer stem cells: Underlying complexity of a simple paradigm. Reproduction **149**: 59-70.

HALES K.H., SPECKMAN S.C., KURREY N.K., HALES D.B. 2014. Uncovering molecular events associated with the chemosuppressive effects of flaxseed: a microarray analysis of the laying hen model of ovarian cancer. BMC Genomics **15**: 709.

INDUMATHI S., HARIKRISHNAN R., RAJKUMAR J.S., SUDARSANAM D., DHANASEKARAN M. 2013. Prospective biomarkers of stem cells of human endometrium and fallopian tube compared with bone marrow. Cell Tissue Res. **3**: 537-549.

ISHII Y., MIKAWA T. 2005. Somatic transgenesis in the avian model system. Birth Defects Res. C. Embryo Today **75**: 19-27.

JOHNSON A.L. 2014. The avian ovary and follicle development: some comparative and practical insights. Turk. J. Vet. Anim. Sci. 38: 660-669.

JUNG J.G., PARK T.S., KIM J.N., HAN B.K., LEE S.D. SONG G., HAN J.Y. 2011. Characterization and Application of Oviductal Epithelial Cells *in vitro* in *Gallus domesticus*. Biol. Reprod. **85**: 798-807.

KAMIHIRA M., KAWABE Y., SHINDO T., ONO K., ESAKA K., YAMASHITA T., NISHIJIMA K., IIJIMA S. 2009. Production

of chimeric monoclonal antibodies by genetically manipulated chickens. J. Biotechnol. **2**: 18-25.

KASPERCZYK K., BAJEK A., JOACHIMIAK R., WALASIK K., MARSZAŁEK A., DREWA T., BEDNARCZYK M. 2012. *In vitro* optimisation of the *Gallus domesticus* oviduct epithelial cells culture. Theriogenology **77**: 1834-1845.

KATO S., TORA L., YAMAUCHI J., MASUSHIGE S., BELLARD M., CHAMBON P. 1992. A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5′-TGACC-3′ motifs acting synergistically. Cell **68**:731-742.

KHUONG T.T., JEONG D.K. 2011. Adipogenic differentiation of chicken epithelial oviduct cells using only chicken serum. *In vitro* Cell. Dev. Biol. Anim. **9**: 609-614.

KWON S., CHOI J.W., JANG H., SHIN S.S., LEE S.K., PARK T.S., CHOI I.J., LEE G.S., SONG G., HAN J.Y. 2010. Production of Biofunctional Recombinant Human Interleukin 1 Receptor Antagonist (rhIL1RN) from Transgenic Quail Egg White. Biol. reprod. **82**:1057-1064.

LEE S.H., GUPTA M.K., HO Y.T., KIM T., LEE H.T. 2013. Transgenic chickens expressing human urokinase-type plasminogen activator. Poult. Sci. **92**: 2396-2403.

LILLICO S.G., SHERMAN A., MCGREW M.J., ROBERTSON C.D., SMITH J., HASLAM C., BARNARD P., RADCLIFFE P.A., MITROPHANOUS K.A., ELLIOT E.A., SANG H.M. 2007. Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc. Natl. Acad. Sci. USA **104**: 1771-1776.

LIM W., BAE S-M., JO G., BAZER F.W., CHOI Y., SONG G. 2015. Prostaglandin D2 synthase related to estrogen in the female reproductive tract. Biochem. Bioph. Res. Co. **456**: 355-360.

LIM W., SONG G. 2013. Discovery of prognostic factors for diagnosis and treatment of epithelial-derived ovarian cancer from laying hens. J. Cancer Prev. **3**: 209-220.

LIVAK K.J., SCHMITTGEN T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**: 402-408.

MOTIANI K., WILLIAMS K., GIRDHAR P.V., MOURAVIEV V., BRACKEN B., KASPER S. 2013. Role of CD44 in Prostate Cancer Stem Cells. J. Urology Suppl. 4S **189**: e104-e105. doi: http://dx.doi.org/10.1016/j.juro.2013.02.1636.

NG A., TAN S., SINGH G., RIZK P., SWATHI Y., TAN T.Z., HUANG R.Y., LEUSHACKE M., BARKER N. 2014. Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. Nat. Cell Biol. **16**: 745-757.

PAIK, D.Y., JANZEN D.M., SCHAFENACKER A.M., VELASCO V.S., SHUNG M.S., CHENG D., HUANG J., WITTE O.N., MEMARZADEH S. 2012. Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serous cancer initiation. Stem. Cells **11**: 2487-97.

PALMITER R.D., WRENN J.T. 1971. Interaction of estrogen and progesterone in chick oviduct development. 3. Tubular gland cell cytodifferentiation. J. Cell Biol. **50**: 598-615.

PARK H.M., SANDERS M.M., SUZUKI T., MURAMATSU T. 2006. An oviduct-specific and enhancer-like element resides at about -3000 in the chicken ovalbumin gene. Biochimie **88**: 1909-1914.

PARK T.S., LEE H.G., MOON J.K., LEE H.J., YOON J.W., YUN B.N.R., KANG S.C., KIM J., KIM H., HAN J.Y., HAN B.K. 2015. Deposition of bioactive human epidermal growth factor in the egg white of transgenic hens using an oviduct-specific minisynthetic promoter. FASEB J. **29**: 2386-2396.

SAWICKA D., CHOJNACKA-PUCHTA L., ZIELIŃSKI M., PŁUCIENNICZAK G., PŁUCIENNICZAK A., BEDNARCZYK M. 2015. Flow cytometric analysis of apoptosis in cryoconserved chicken primordial germ cells. Cell. Mol. Biol. Lett. **20**: 143-159.

SCOTT B.B., LOIS C. 2005. Generation of tissue-specific transgenic birds with lentiviral vectors. PNAS **102**: 1643-1647.

SIWEK M., SŁAWIŃSKA A., ŁAKOTA P., GRAJEWSKI B., WAWRZYŃSKA M., WIŚNIEWSKA E., PŁAWSKI A., SŁOMSKI A., BEDNARCZYK M. 2010. Identification of the Rate of Chimerism of Different Tissues with Microsatellite Markers in Chicken Chimeras. Folia biol. (Kraków) **58**: 257-263.

SNEGOVSKIKH V., MUTLU L., MASSASA E., TAYLOR H.S. 2014. Identification of putative fallopian tube stem cells. Reprod Sci. **21**: 1460-1464.

STADNICKA K., MARSZAŁEK A., KOZŁOWSKA I., WALASIK K., BODNAR M., BAJEK A., POROWIŃSKA D., DREWA T., BEDNARCZYK M. 2014. The Expression of p63 and Ck HMW in Magnum and Infundibulum of *Gallus domesticus* Oviduct. Folia Biol. (Kraków) **3**: 179-185.

TREVIÑO L.S., GILES J.R., WANG W., URICK M.E., JOHNSON P.A. 2010. Gene expression profiling reveals differentially expressed genes in ovarian cancer of the hen: support for oviductal origin? Horm. Cancer **4**: 177-86.

WAWRZYŃSKA M., BEDNARCZYK M., ŁAKOTA P., LUBISZEWSKA M. 2008. Influence of Electroporation on Chicken Blastoderm Cell Viability *in vitro*. Folia Biol. (Kraków) **56**: 197-201.

YAMANO S., DA J., MOURSI A.M. 2010. Comparison of Transfection Efficiency of Nonviral Gene Transfer Reagents. Mol. Biotechnol. **46**: 287-300.