

## The Expression of p63 and Ck HMW in Magnum and Infundibulum of *Gallus domesticus* Oviduct\*

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The potential for proliferation and differentiation has a critical meaning in terms of the long-term *in vitro* culture of oviductal target cells. Therefore, it is important to characterize the oviduct epithelial cells, using approved markers. There is scarce data describing the epithelial cells lining the avian oviduct, most of it referring only to the magnum section of the oviduct. This study presents a comprehensive analysis of both magnum and infundibulum tissues, as the most preferred sources of epithelial cells for research on production of recombinant proteins in oviducts of birds. The main objective was to evaluate the expression of p63 and high molecular weight cytokeratins (anti-p63 antibody and anti-High Molecular Weight Cytokeratins) in epithelial cells (EC) of 2 oviduct sections: magnum (proximal and middle) and infundibulum (distal). IHC analysis and western blotting were performed using the mouse monoclonal anti-p63 antibody and anti-Ck HMW. Immunoreactivity was quantified based on the Remmele - Stegner scoring system (0-12). The expression of p63 in nuclei of luminal cells was significantly higher in the infundibulum ( $P < 0.05$ ), compared to the magnum section. Cytokeratins were also highly expressed in the infundibulum, but the difference was non-significant. These findings reveal new characteristics of the oviduct EC and designate the location of the source of cells in the oviduct tissue for *in vitro* culture.

Key words: Oviduct, avian, progenitor cells, p63, Ck HMW.

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Avian species are important model organisms in terms of the production of therapeutic proteins and various methods to study transgenesis of birds, among which mainly germ cells are used, have been developed recently (CHOJNACKA-PUCHTA *et al.*

2012; GWONGHA & HAN 2011; JUNG *et al.* 2011). However, obstacles such as predisposition of cells to artifacts during their retrieval and handling (VAN SOOM *et al.* 2010) affect the control of the differentiation process in long term culture (JUNG

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*et al.* 2010). Apart from being useful in the bioreactor approach, oviduct tissue serves as a good model for biomedical research by means of remarkable analogy between the ovulatory cycles of women and hens (JOHNSON & GILES 2013) and the spontaneous adenocarcinomas occurring in the latter (TREVINO *et al.* 2010; RICCI *et al.* 2013).

Recently, attention has turned towards adult multipotent stem cells (DAVIES & FAIRCHILD 2011) which are well described in mammals, but not in birds (HEO *et al.* 2012). Together with the discovery of progenitor and stem cell niches, vast opportunities for biomodeling in clinical trials and *in vitro* studies, including toxicological evaluations, have appeared (GATTENGO-HO *et al.* 2012). Stem cell niches provide a suitable environment and are necessary to maintain homeostasis of colonizing stem cells (DREWA *et al.* 2009). Prospective use of avian oviduct stem cells requires the identification of specific markers allowing for precise recognition and isolation of adult stem cells. Two major groups of stem cell markers of epithelial origin are currently available, the first of which are intracellular proteins (cytokeratins) and the second are cell surface proteins. It is convenient that most primary antibodies applied in immunohistochemical (IHC) studies are known to cross-react with avian tissues (MANAROLLA *et al.* 2011) making them available for chicken studies. YANG *et al.* (1999) reported that the transcription factor p63 is critical for maintaining progenitor cell populations necessary to sustain epithelial development and morphogenesis. It is also highly expressed in embryonic ectoderm and in the nuclei of basal regenerative cells of many adult epithelial tissues including skin, prostate and urothelia (WESTFALL & PIETENPOL 2004). Monoclonal anti-Ck HMW (high-molecular weight cytokeratins) are a mixture of antibodies which can react with several cytokeratins (Ck 1-5-10-14), indicating the presence of highly proliferative myoepithelial cells in the epithelial basal cell layer. We demonstrate how the cells of infundibulum and magnum sections of hen oviducts react with epithelial cell markers.

## Material and Methods

### Experimental animals

The birds used in experiments were randomly selected, hybrid Tetra SL laying hens (n=5, 33-week-old), not stimulated by hormones, sacrificed according to the regulations of Polish Local Ethical Commission and the Directive 2010\_63\_UE\_PL. The hens were in the egg production period and the oviduct tissue after egg mass passage was used for this study.

### Isolation of the oviduct tissue, immunohistochemistry and western blotting

Three different portions of the oviduct from each hen, i.e. the middle magnum, the upper magnum and the infundibulum neck, were dissected and washed twice in PBS (H15-002, PAA, Immuniq, Zory, Poland) with penicillin/streptomycin solution 1:100 (P11-010, PAA, Immuniq, Zory, Poland). Afterwards the tissue sections were fixed in 10% (v/v) buffered formalin, subjected to a routine dehydration procedure, placed in xylene and embedded in paraffin. IHC assay was conducted using mouse mAb against p63 (ab59691 clone: 4A4RTU, Abcam, STI, Poznan, Poland; dilution 1:10) and the monoclonal anti-Ck to HMW (clone: 34βE12, DakoPolska Sp. z o.o., Gdynia, Poland; dilution 1:50). The epitopes were unmasked using the Epitope Retrieval Solution (high pH) with PT-Link (DakoPolska Sp. z o.o., Gdynia, Poland) and incubated with anti-p63 and anti-Ck HMW primary antibodies for 30 minutes at RT or 37°C, respectively. The antibody complexes were detected by the EnVision Anti-Mouse HRP Labelled Polymer (DakoPolska Sp. z o.o., Gdynia, Poland) with DAB as chromogen and the presented antigens were identified under a light microscope as brown reaction products at original magnification: × 200. The levels of expression were evaluated in five randomly selected areas for each antigen and calculated according to the REMMELE & STEGNER (1987) scale (IRS: 0-12 points), based on the intensity of the stain reaction (SI) and the percentage of positive cells (PP).

Also, protein samples were isolated from the homogenized oviduct tissue for western blotting analyses. SDS-PAGE was performed using OGITA and MARKERT method (1979). Anti-p63 and anti-Ck to HMW (the same as for IHC) were used for incubation with the anti-mouse secondary Ab conjugated with alkaline phosphatase (ab6790, Abcam, STI, Poznan, Poland; dilution 1:200).

### Epithelial oviduct cell culture

In order to assess the growth of the oviduct cells *in vitro*, the cells were isolated from the same oviduct sections as earlier evaluated with Ck HMW and p63 and cultured according to the method described by KASPERCZYK *et al.* (2012), in three repetitions. Briefly, the oviduct sections were cleaned from remaining connective tissue, minced and digested with 1mg/ml Collagenase P (11 213 857 001, Roche Polska Sp. z o.o., Warszawa, Poland) for 30 min with shaking. Approximately  $0.5 \times 10^6$  cells per well were seeded onto non-treated polystyrene 24-well culture plates (353935, Becton Dickinson, DIAG-MED, Warszawa, Poland). The cells were maintained in defined serum-free medium dedi-

cated for growth of keratinocytes (00192151 and 00192152, Lonza S.A., Warszawa, Poland) with the addition of Mycokill (AB P11-016, PAA, Immuniq, Zory, Poland) at 37°C in a 5% CO<sub>2</sub>-humidified incubator. After 7 days, the primary epithelial colonies were counted and photographed. The results were expressed as the mean number of colonies which were able to develop from the cells inocula.

Statistical procedure

The Shapiro-Wilk test was used to assess the normality of data distribution. Multiple comparisons for all pairs were performed with two-way ANOVA and Tukey’s HSD post hoc test. Differences in the expression of antibodies (Cks and p63) in the three oviduct sections are shown as means (Stat Soft® Polska v.8, Stat Soft Polska Sp. z o.o., Krakow, Poland). For these tests, P<0.05 was considered significant.

Results

p63 and CkHMW expression by IHC analysis

As shown in Fig. 1, the p63 factor level was highly expressed in the nuclei of tubular epithelial cells lining the crypts of infundibulum tissue (mean IRS: 3.2, P<0.05) whereas in magnum the level of expression was low (mean IRS<1.0). The membranous and cytoplasmic staining reactions

of Cks were the highest in the infundibulum (mean IRS: 12.0). Differences in Ck HMW expression were not significant between the proximal (upper) magnum (mean IRS: 7.2) and the middle magnum (mean IRS: 5.2; Fig. 1). Microphotographs (Fig. 2) show the distribution of the luminal oviduct cells expressing p63 and Ck HMW. A strongly positive reaction was observed in the infundibulum (Fig. 2. Panels A and C) whereas specimens of magnum demonstrated a weak staining reaction.

p63 and CkHMW expression by western blotting

The western blotting analysis (Fig. 3) revealed post-electrophoresis bands corresponding to p63 (77 kDa) only in the samples derived from infundibulum tissue, whereas Ck HMW were expressed both in infundibulum and magnum. Two polypeptides can be clearly distinguished, probably corresponding to the cytokeratins Ck 5 and Ck 14 (50 kDa and 58 kDa bands, respectively). There was a gradual reduction in the expression of markers from infundibulum, through proximal to middle magnum (Fig. 2. Panels B and D).

Epithelial oviduct cell culture

Cells isolated from the infundibulum of the oviduct formed confluent, adherent colonies of epithelial-like morphology, suitable for cultivation *in vitro* (Fig. 4), whereas the cells isolated from the middle magnum scarcely developed *in vitro*.

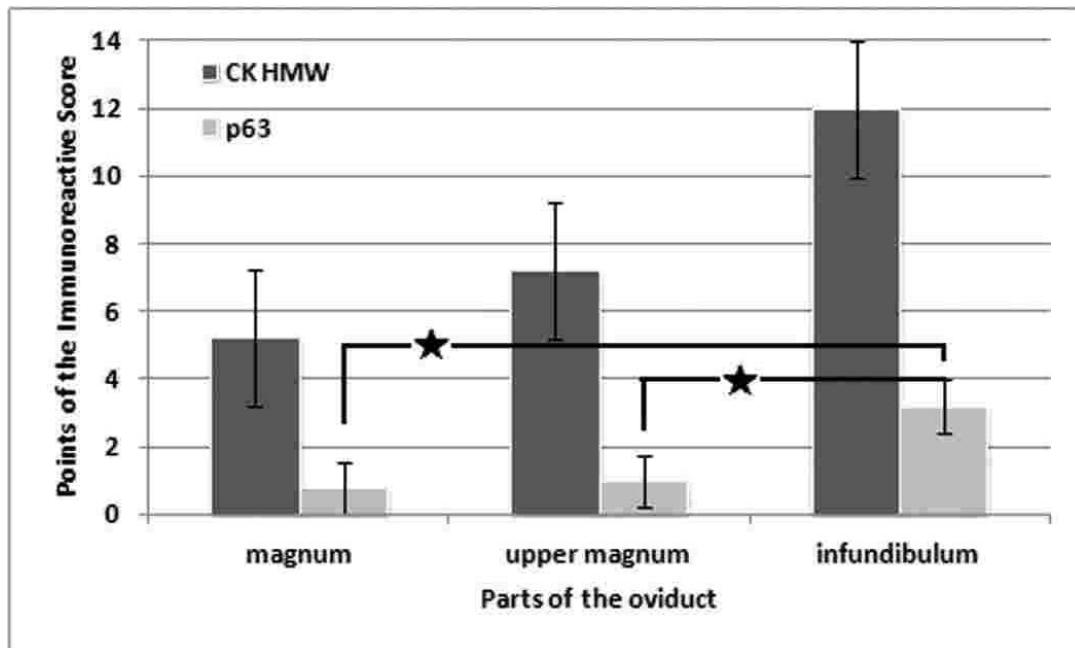


Fig. 1. Mean immunoreactive score of epithelial cells positive to anti- p63 and Ck HMW in the oviduct (middle) magnum, upper (proximal) magnum and distal infundibulum. ANOVA with Tukey-HSD post-hoc shows differences (P<0.05) in expression of p63 between the infundibulum and magnum sections (\*).

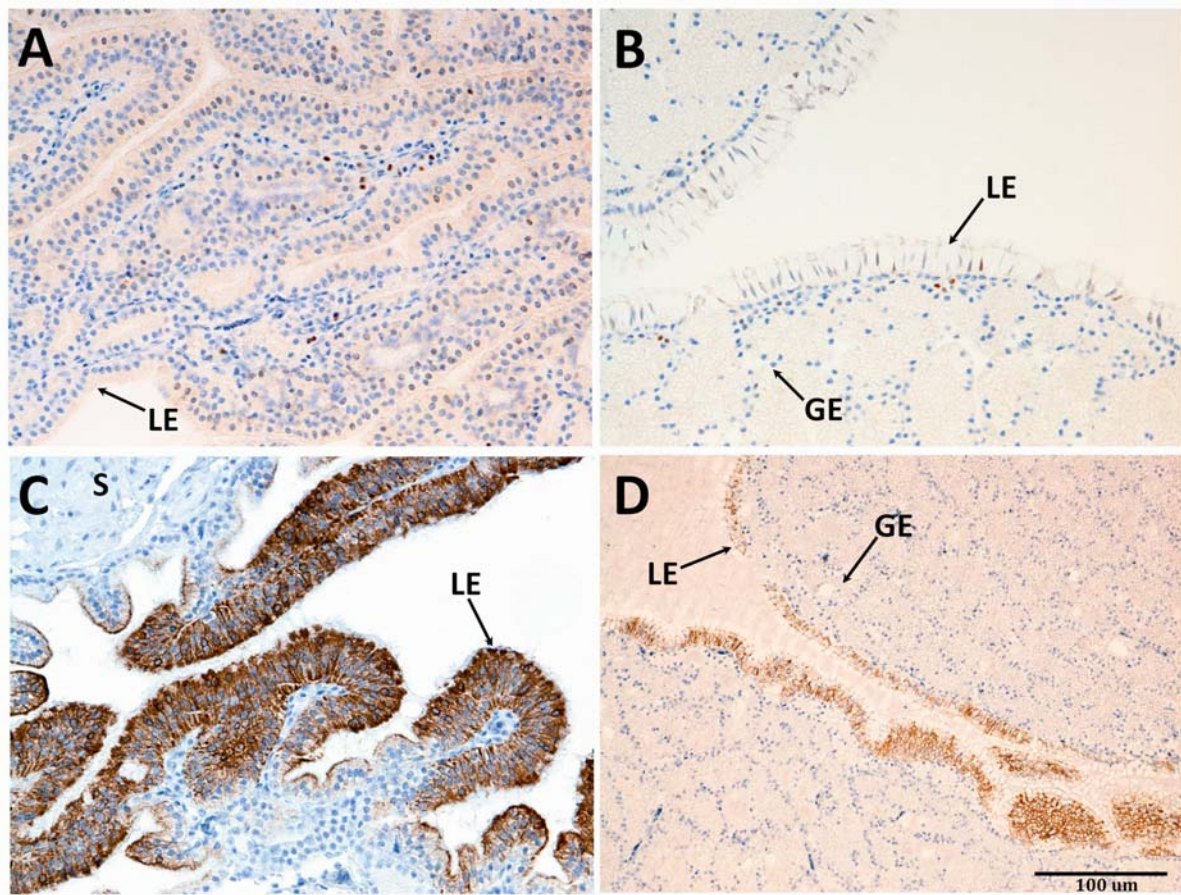


Fig.2. Microphotographs after IHC stain with anti- p63 and anti- Ck to HMW antibodies: diffuse and intense expression of p63 in infundibulum nuclei (A); multifocal, moderate expression in magnum nuclei (B); diffuse, distinct cytoplasmic/membranous expression of Cks in the infundibulum crypts (C); weak, focal expression in magnum (D); original magnification: x 200. LE – luminal Epithelium, GE – Glandular Epithelium, S – Stroma.

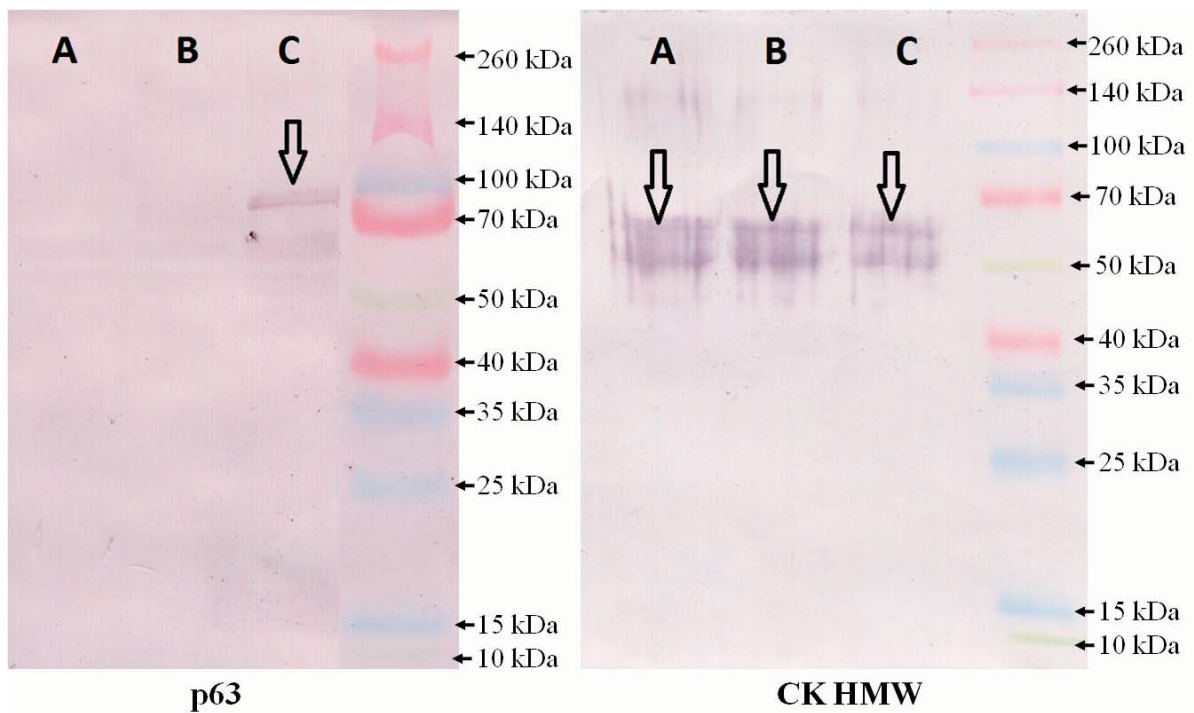


Fig. 3. Western blotting patterns with anti-p63 and anti-Ck HMW antibodies: A – middle magnum; B – proximal magnum (region of transition between magnum and infundibulum); C – distal infundibulum. The arrows indicate banding patterns representing p63 (77kDa) and Ck HMW (50kDa and 58kDa). Spectra Multicolor Broad Range Protein Ladder 26623 (SM 1842) was used as a molecular weight standard.

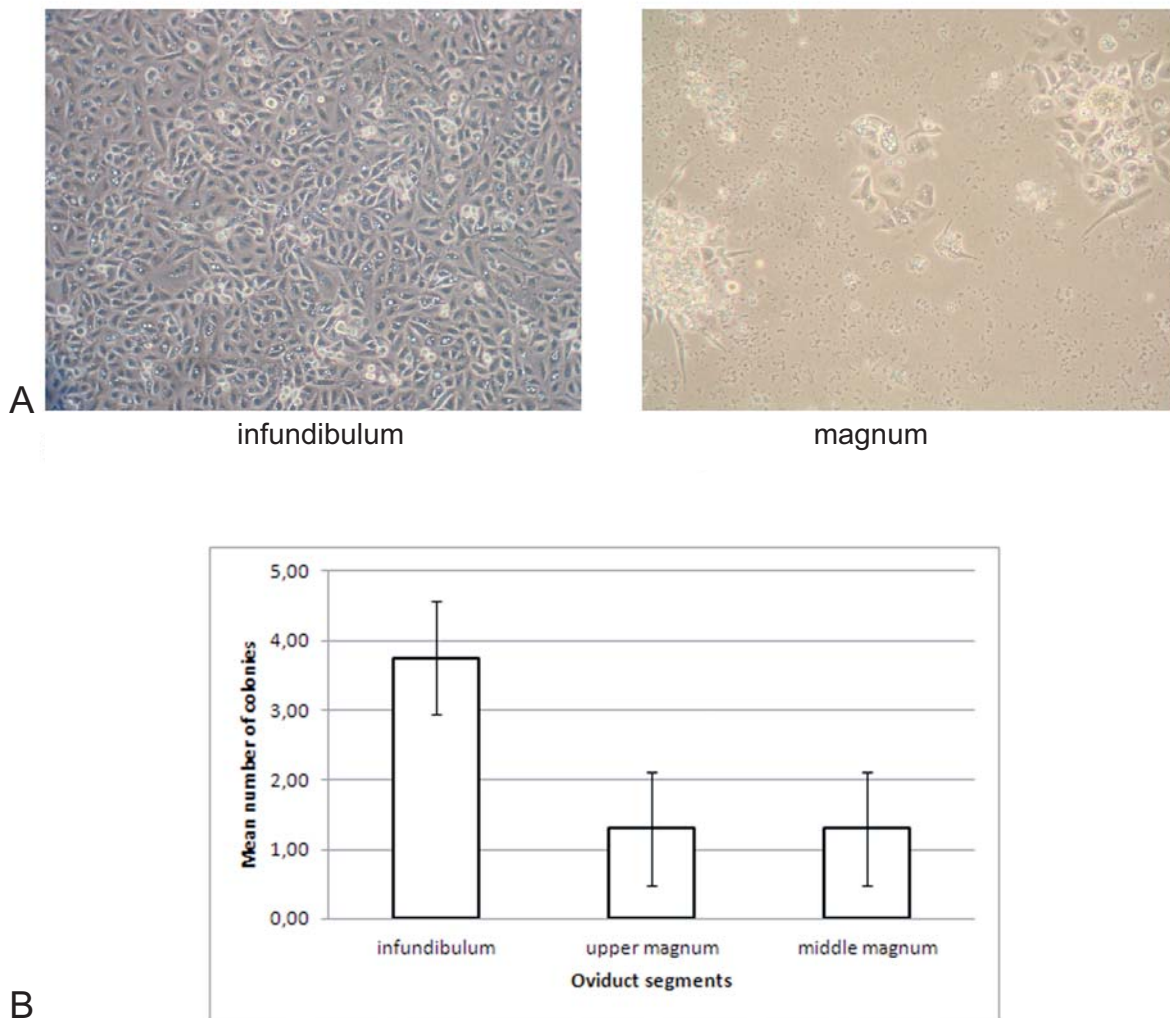


Fig. 4. A – microphotographs of representative epithelial-like cell colonies developed in serum-free medium; taken under inverted microscope (ECLIPSE E800, Nikon), original magnification:  $\times 100$ ; B – number of cell colonies formed from different oviduct sections after 7 days of culture *in vitro*. Seeding number:  $0.5 \times 10^6$  cells. Values are the means  $\pm$  SD of at least three independent experiments.

## Discussion

The p63 protein belongs to the family of transcription factors of the suppressor protein p53 and is responsible for epithelial differentiation. It was described by YANG *et al.* (1999) for the first time whilst subsequent studies of PELLEGRINI *et al.* (2001), INCE *et al.* (2002) and BARBIERI & PIETENPOL *et al.* (2006) suggested an important role for this protein in execution of biological functions by epithelial cells, even suggesting the status of a progenitor cell marker (KAI-HONG *et al.* 2007). The p63 protein is found in the basal layer of various epithelia (FEIL *et al.* 2008). We show p63 positive epithelial cells in the infundibulum and magnum of the hen oviduct which might suggest that the infundibulum carries not only fully functional, but also proto-differentiated/ precursory epithelial cells of the oviduct, in other words cells that are not terminally differentiated but arrested in a quiescent state

characteristic of most adult stem cell populations (BOWEN *et al.* 2009). We also came to this conclusion in a previous study (KASPERCZYK *et al.* 2012), as the cells isolated from infundibulum were easier to maintain *in vitro*, propagated faster compared to cells isolated from the magnum and were able to reach confluence or form compact epithelial-like colonies during cultivation (Fig. 4). The oviduct epithelium undergoes a renewal process due to the incessant ovarian cycle and the secretion of cells of infundibulum and magnum are replaced by proto-differentiated cells, still poorly understood. This should be further investigated using markers of multipotency and differentiation process analysis (adipogenic, chondrogenic, osteogenic) in cultured infundibulum and magnum cells. The microphotograph (Fig. 2) shows the distribution of cells expressing p63 and Ck HMW in luminal epithelium and partially between the basal lamina and glandular cells in the oviduct lining. Earlier,

KURITA *et al.* (2004) stated that p63 determines the developmental fate of ductal epithelial cells and is expressed in basal cells of cervical epithelium. The same group conducted studies on uterus, classified as an epithelio-mesenchymal organ and indicated that p63-expressing cells can retain developmental plasticity in the adult tissue as certain types of stem cells (KURITA 2011). Earlier, SENOO *et al.* (2007) demonstrated that p63 was strongly expressed in epithelial cells with clonogenic and proliferative capacity.

In this study the western blotting post-electrophoresis bands corresponding to anti-p63 (77kDa) were present only in infundibulum derived samples, whereas Ck HMW were present both in infundibulum and magnum of the oviduct. In our opinion, isolation of the population of cells expressing this marker might contribute to efficient colony-formation of oviduct epithelial cells cultured *in vitro*.

There is insufficient data regarding the oviduct cell renewal process in birds and recently JEONG *et al.* (2013) described the degeneration and recovery of oviduct epithelia during the remodeling process at the end of laying cycles using EMT (epithelial-to-mesenchymal transition) and proliferation markers including cytokeratins. The number of colonies grown *in vitro* accurately resembles the quantity of mesenchymal stem cells in bone marrow (DIGIROLAMO *et al.* 1999). In a pioneer study (FRIEDENSTEIN *et al.* 1974) it was shown that MSCs grown in low density were able to constitute colonies descending from single cells. In previous studies we observed morphologically miscellaneous colonies consisting of spindle-shaped and cubical cells (KASPERCZYK *et al.* 2012), the latter of which were considered elsewhere as typical for colonies grown from single cells identified as clonogenic (BOBIS *et al.* 2006; DIGIROLAMO *et al.* 1999).

In summary, to effectively establish cell culture *in vitro*, a certain population of precursory cells (DREWA *et al.* 2008; DREWA & STYCZYNSKI 2007) should be isolated from proximal oviduct sections including distal infundibulum and proximal magnum, followed by the analysis of multipotency markers. The main purpose for this is to identify useful sources of tissue to develop advanced *in vitro* models addressing cell biology, veterinary and transgenic bioreactor studies.

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