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Expression of transcription factors during area pellucida formation in intrauterine chicken embryos

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ABSTRACT Initial embryological development in avian species, consisting of cleavage and area pellucida formation, occurs prior to oviposition. In chickens, the first lineage segregation is known to occur during the last 10 hours of intrauterine development, a finding which has primarily been identified on the basis of morphological perspectives. We traced the early expression of the transcription factors *NANOG*, *POUV* and *EOMES* at Eyal-Giladi and Kochav (EGK) stages VI through X using *in situ* hybridization. At EGK.VI, *NANOG* and *EOMES* were heterogeneously expressed in a salt-and-pepper manner. From EGK.VIII to EGK.X, *NANOG*- or *EOMES*-positive cells were predominantly located in the epiblast or area opaca regions, respectively. *POUV*-expressing cells were found in the upper layer at EGK.VIII. After oviposition, *POUV* mRNA was strongly expressed in the epiblast, but weakly expressed in the hypoblast at EGK.X. Furthermore, *NANOG*- and *POUV*-negative cells were located in the subgerminal cavity where layer reduction occurs during area pellucida formation. Those cells were larger and did not seem to contribute to epithelialization until EGK.X. Our results on the spatiotemporal expression of transcription factors contribute to a greater understanding of the dynamic process of intrauterine development in chickens.

KEY WORDS: *chicken, intrauterine development, area pellucida formation, transcription factor*

Avian species have great value as *ex vivo* models for both developmental and application-oriented studies, enabling the monitoring of organogenesis and lineage differentiation, which have made great contributions to embryology (Stern, 2005). Most studies of avian development have been limited to primitive streak formation and gastrulation after oviposition (Streit *et al*., 2000, Skromne and Stern, 2001, Torlopp *et al*., 2014). However, the initial events of avian embryogenesis that occur prior to oviposition are unclear, owing to the practical difficulties of harvesting pre-oviposited embryos.

Pre-ovipositional development was morphologically established according to the criteria of Eyal-Giladi and Kochav (EGK) (Eyal-Giladi and Kochav, 1976, Kochav *et al*., 1980). Based on these criteria, the intrauterine period before oviposition is divided into ten stages comprising the periods of cleavage and area pellucida formation. During the first six stages (EGK.I to EGK.VI), called the cleavage period, rapid asymmetric cellularization and layering occur. Subsequently, early lineage segregation, with anterior-posterior axis formation and layer reduction, occurs during the period of area pellucida formation (EGK.VII to EGK.X) (Sheng, 2014).

During mouse embryogenesis, transcription factors such as nanog homeobox (NANOG) and POU domain class 5 transcription factor 1 (POUV) are involved not only in fundamental events such as zygotic genome activation (ZGA), but also in the early lineage segregation of cells in the inner cell mass (ICM) (Mitsui *et al*., 2003, Lee *et al*., 2013b, Leichsenring *et al*., 2013). NANOG is also a critical factor in epiblast specification, as well as ICM specification (Mitsui *et al*., 2003), whereas eomesodermin (EOMES) is a known extraembryonic marker in mice and chicks (Pernaute *et al*., 2010). However, the expression profiles of these genetic modulators during intrauterine development have not been investigated in birds. Therefore, we investigated

Abbreviations used in this paper: DIG, digoxigenin; EGK, Eyal-Giladi and Kochav; EOMES, eomesodermin; ICM, inner cell mass; NANOG, nanog homeobox; POUV, POU domain class 5 transcription factor 1; WL, white leghorn; ZGA, zygotic genome activation.

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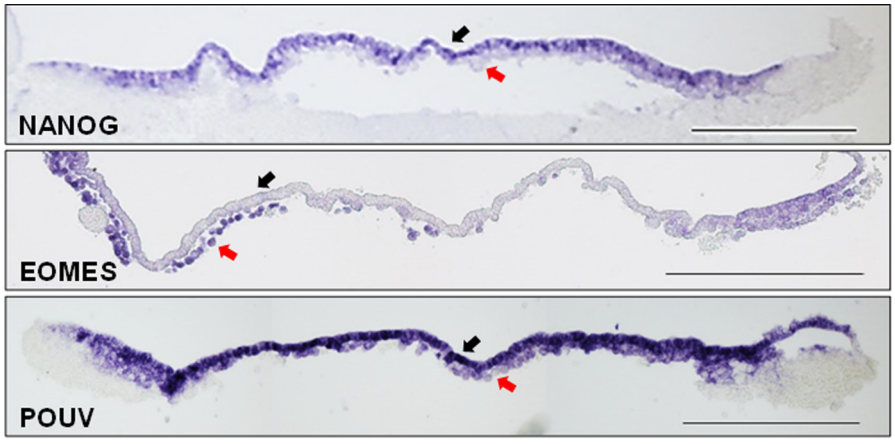

Fig. 1. Expression of *NANOG*, *EOMES* and *POUV* in longitudinal sections of EGK stage X blastoderm by *in situ* hybridization. NANOG *is strongly expressed in the epiblast of the area pellucida and area opaca.* EOMES *is expressed in the area opaca and hypoblast.* POUV *is more strongly expressed in the epiblast than in the hypoblast. Black arrows, epiblast; red arrows, hypoblast. Scale bars, 500 μm.*

the spatiotemporal expression patterns of the pluripotency- and extraembryonic determination-related transcription factors *NANOG*, *POUV*, and *EOMES* during area pellucida formation in chickens using *in situ* hybridization.

Results

Tracing of chicken NANOG and EOMES expression from EGK.VI to EGK.X

With the advent of area pellucida formation at EGK.VII, the multilayered blastoderm is extensively transformed into a single- or bi-layered epithelial blastoderm by EGK.X (Eyal-Giladi and Kochav, 1976, Kochav *et al.*, 1980). We assessed the expression of transcription factors during this period. At EGK.X, *NANOG* showed stronger expression in the epiblast than in the hypoblast, whereas *EOMES* was expressed in the hypoblast and the area opaca but absent in the area pellucida (Fig. 1). Before oviposition, *NANOG* mRNA showed a heterogeneous expression pattern in all cell layers at EGK.VI and EGK.VII (Fig. 2A and Fig. 2B). While *NANOG*-positive cells (red arrows) were well distributed throughout all layers, *NANOG*-negative cells (black arrows) were mainly located in the middle and lower layers. At EGK.VIII, the uppermost layer was composed of *NANOG*-positive cells, whereas *NANOG*-negative cells, which were relatively larger than *NANOG*-positive cells, were located in the lower layer or detached from the upper layer (Fig. 2C, black arrows). Furthermore, the expression pattern of *EOMES* was similar to that of *NANOG* at EGK.VI (Fig. 3A) before definite area pellucida formation. As development progressed, *EOMES* was predominantly expressed in the future area opaca region (Fig. 3B). Over the course of area pellucida formation, *NANOG* and *EOMES* showed mixed expression from EGK.VI–VII, then switched to strong expression in the cells of the upper or outer layer, respectively, at EGK.VIII.

Chicken POUV expression and large detached cells during area pellucida formation

At EGK.X, *POUV* mRNA was strongly expressed in the epiblast of the area pellucida and the area opaca, but weakly expressed in the hypoblast (Fig. 1). Accordingly, we next investigated *POUV* expression during area pellucida formation. While layer reduction was occurring at EGK.VIII, the cells in the uppermost layer were

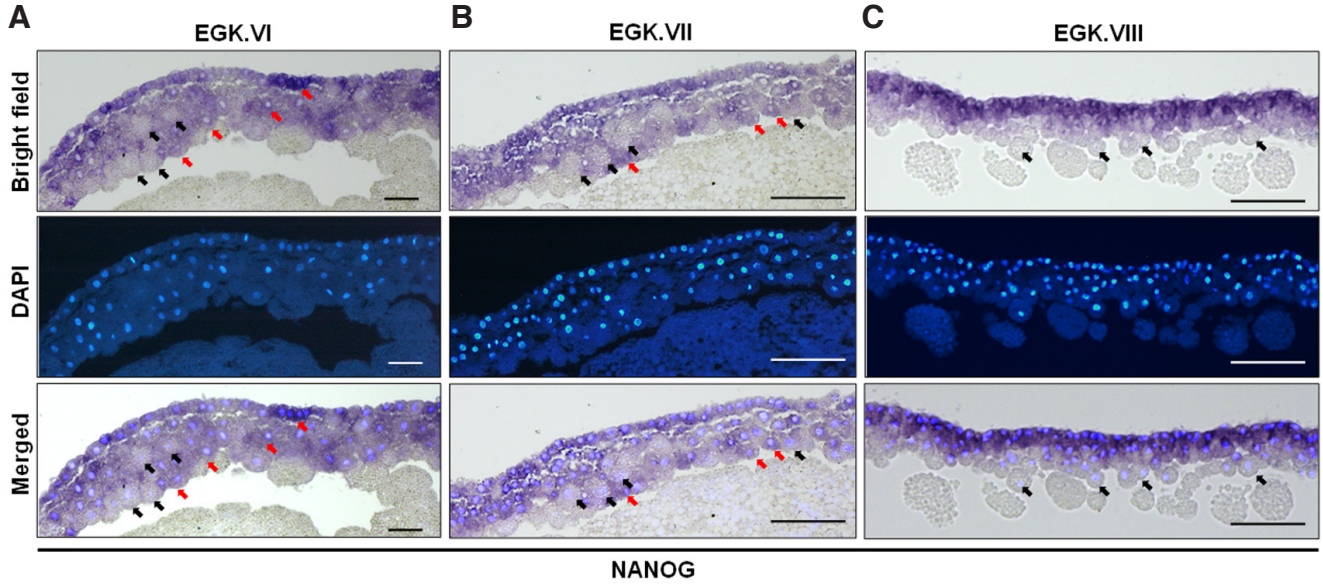

Fig. 2. Expression of *NANOG* during the period of area pellucida formation by *in situ* hybridization. *Magnified views of* NANOG *expression at EGK.VI* **(A)** *and EGK.VII* **(B)** *indicate a mixed distribution of* NANOG*-negative (black arrows) and -positive cells (red arrows) at these stages.* **(C)** *At EGK.VIII, most of the* NANOG*-negative cells are found in the lowermost cell layer, whereas* NANOG*-positive cells are found in the uppermost layer. Scale bars, 100 μm.*

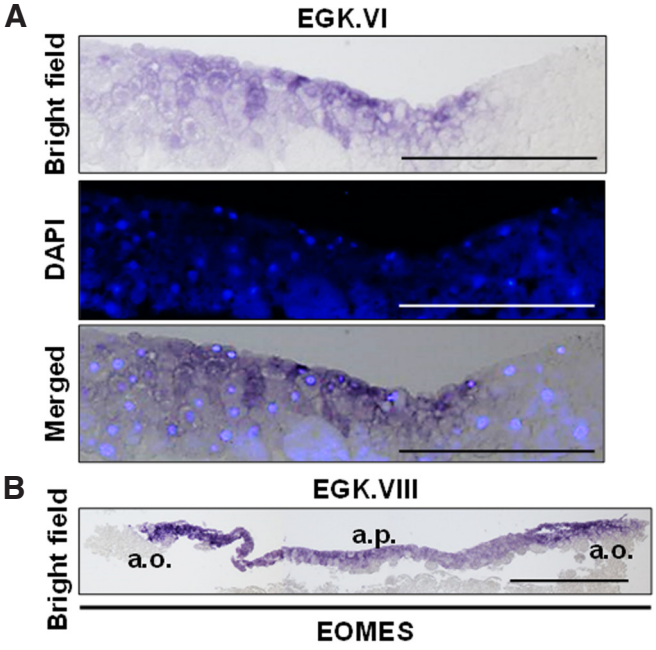

Fig. 3. Expression of *EOMES* during area pellucida formation at EGK.VI and EGK.VIII by *in situ* hybridization. *At EGK.VI* **(A)**, EOMES *expression is dispersed throughout the longitudinal sections, but at EGK. VIII* **(B)**, *it is more abundant in the area opaca than in the area pellucida. Scale bars, 500 μm.*

POUV-positive, whereas those in the lower layers were *POUV*-negative, similar to the pattern observed for *NANOG* expression (Fig. 4A; black arrows). Compared with the *POUV*-positive cells, the *POUV*-negative cells were clearly larger and were located in the subgerminal cavity (Fig. 4A). In addition, at low magnification, cell detachment was clearly observed in the anterior area of EGK.VIII sections (Fig. 4A). After area pellucida formation was complete at EGK.X, some detached cells in the subgerminal cavity had nuclei (Fig. 4B; white arrows), while other cells fused with the yolk (Fig. 4B; yellow arrows) and were obviously distinct from epiblastic and hypoblastic cells (Fig. 4B; blue and red arrows). We also measured the diameter of the detached cells (Fig. 4C; white arrows) and of the cells in the uppermost layer (Fig. 4C; blue arrows) at EGK.VIII. The detached cells were significantly larger (31.17 ± 9.45 mm, n = 20) than the cells in the uppermost layer (9.42 ± 2.83 mm, n = 20) (Fig. 4C). These results indicate that *NANOG*- and *POUV*-negative cells were observed during area pellucida formation, and were larger than the cells in the upper layer.

Discussion

The cell layers in chicken embryos increase rapidly from EGK.I to EGK.VI via cell division, and subsequently decrease to constitute single- and bi-layered EGK.X embryos. Simultaneously, the first lineage specification of the epiblast and hypoblast, and area opaca formation, occur during intrauterine development in the chicken (Eyal-Giladi and Kochav, 1976, Kochav *et al.*, 1980). However,

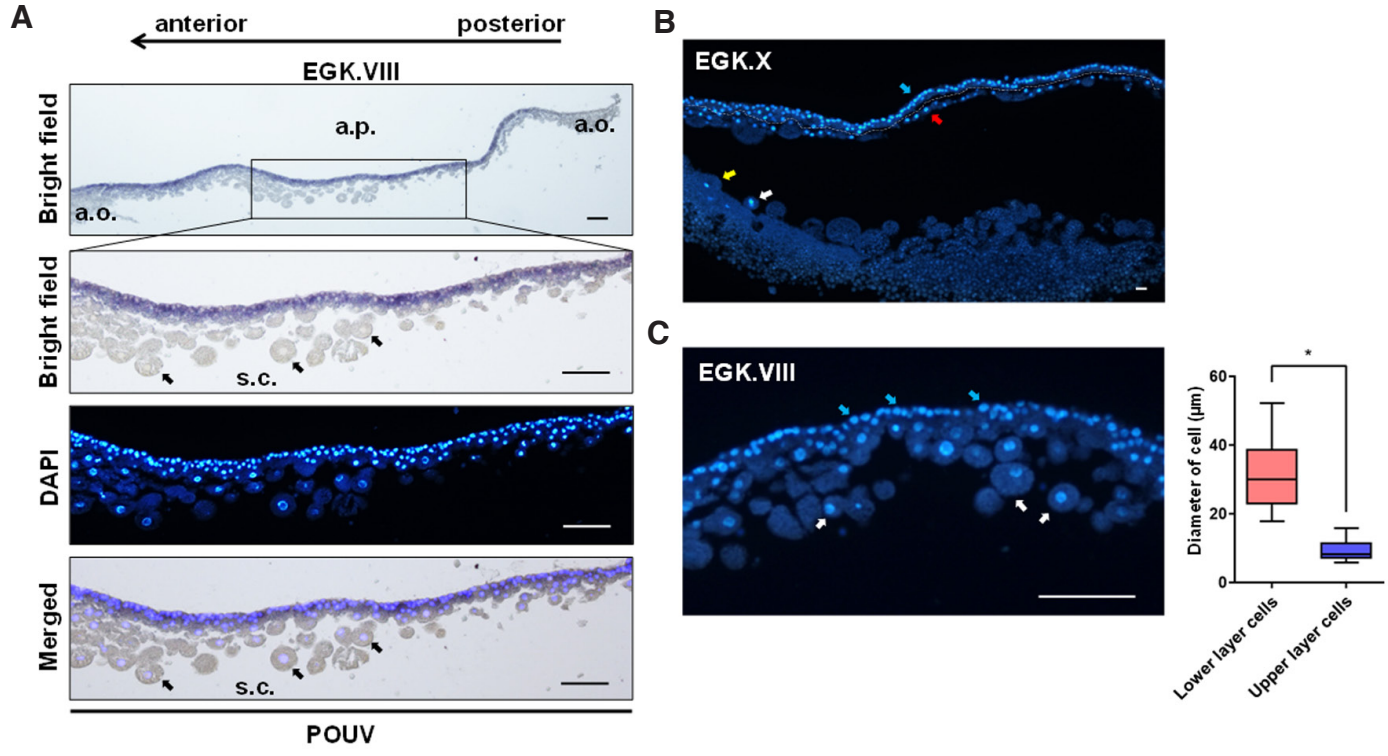

Fig. 4. Expression of *POUV* by *in situ* hybridization and the morphology of lower layer cells. (A) POUV *expression at EGK.VIII. Almost all* POUV*-positive cells are located in the uppermost layer, whereas* POUV*-negative cells (black arrow) are detached under the epiblastic layer. a.p., area pellucida; a.o., area opaca; s.c., subgerminal cavity.* **(B)** *EGK.X blastoderm showing the epiblast (blue arrow) and hypoblast (red arrow). The white and yellow arrows indicate cells located in the subgerminal cavity and fused with the white yolk of the blastoderm, respectively.* **(C)** *The diameter of cells in the EGK.VIII blastoderm. White arrows indicate the detached lower layer cells, which have a greater volume of cytoplasm and larger nuclei than cells of the uppermost layer (blue arrows). Significant difference between groups are indicated as* *$p < 0.05$ *using the Student t-test. Scale bar, 100 μm.*

these processes have primarily been studied in terms of their morphological aspects. Here, we examined the spatiotemporal expression of three transcription factors, *NANOG*, *POUV*, and *EOMES*, during these processes.

During intrauterine stages EGK.VI–VII, *NANOG* and *EOMES* showed similar heterogeneous expression patterns in the embryos. These results indicate that the precursors of the epiblast and hypoblast may be predetermined in a salt-and-pepper manner, consistent with a previous report in zebra finch (Mak *et al.*, 2015). It has been reported that ZGA occurs between late EGK.II and early EGK.III, based on the phosphorylation of the RNA polymerase II C-terminal domain (Nagai *et al.*, 2015), indicating that zygotic expression of transcription factors may diversify cell states before morphological segregation. As development progressed to EGK.VIII, *NANOG*- or *EOMES*-positive cells were predominantly arranged in the upper layer or in the future area opaca, respectively. Finally, at EGK.X, *NANOG* expression was observed in the epiblast, whereas *EOMES* expression was limited to the area opaca and hypoblast. Considering the rapid layer reduction that occurs during these stages, the changes in expression patterns in different embryonic regions may indicate cellular rearrangement, similar to the radial intercalation observed in frog and fish embryos (Keller, 2002). On the other hand, *NANOG*-negative cells were detached from the upper layer at EGK.VIII, and similar cells in the subgerminal cavity expressing *POUV* were clearly observed in this study. According to previous studies, this unique cell detachment phenomenon is involved in layer reduction in the multilayered chicken blastoderm, and related to axis formation, as it occurs in the future posterior-to-anterior direction (Fabian and Eyal-Giladi, 1981, Kochav *et al.*, 1980).

NANOG and POUV are required for the maintenance of pluripotency and self-renewal in embryonic stem cells in chickens as well as in mammals (Lavial *et al.*, 2007). In this study, none of these transcription factors were detected in the significantly larger lower layer cells than those of the uppermost layer cells at EGK.VIII. These *NANOG*- and *POUV*-negative cells, lacking pluripotency, were likely not involved in epithelialization or in contributing to future blastodermal cells. However, further detailed elucidation, including live imaging is required to understand the status of these *NANOG*- and *POUV*-negative cells during the formation of the area pellucida.

Taken together, our findings demonstrate the spatiotemporal expression of three transcription factors (*NANOG*, *POUV*, and *EOMES*) during pre-ovipositional stages in chicken. *NANOG* and *EOMES* were heterogeneously expressed in a salt-and-pepper manner at EGK.VI, and predominantly located in the epiblast or area opaca regions, respectively at EGK.VIII to EGK.X. *POUV* expression was found in the upper layer at EGK.VIII, and became strong in the epiblast at EGK.X. Furthermore, *NANOG*- and *POUV*-negative cells were located in subgerminal cavity where layer reduction occurs during area pellucida formation. Our results contribute to a greater understanding of the dynamic process of intrauterine development in chickens.

TABLE 1

PRIMER SEQUENCES USED IN THE *IN SITU* HYBRIDIZATION ANALYSIS

Gene	Forward (5'→3')	Reverse (5'→3')
cPOUV	GCCAAGGACCTCAAGCACAA	ATGTCACTGGGATGGGCAGA
cNANOG	CAGCAGACCTCTCCTTGACC	AAGCCCTCATCCTCCACAGC
cEOMES	TGACTGAAGATGGCGTTGAG	CTTCAGAGAAGCCTGGAGGA

Materials and Methods

Experimental animals and animal care

The care and experimental use of chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). Chickens were maintained according to a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation were in adherence with the standard operating protocols of our laboratory.

Collection of intrauterine embryos

Intrauterine eggs were retrieved from white leghorn (WL) hens by an abdominal massage technique as previously reported (Lee *et al.*, 2013a). Briefly, the abdomen was pushed gently until the shell gland was exposed. The surface of the shell gland expanded when an egg was located there for eggshell formation. After this expansion of the shell gland, the intrauterine egg was gently moved toward the cloaca via massage until it was released. Intrauterine embryos were classified according to the criteria of EGK (Eyal-Giladi and Kochav, 1976, Kochav *et al.*, 1980). The harvested blastoderms were fixed in 4% paraformaldehyde in PBS for subsequent experiments. Fertility and abnormalities were determined according to the morphology.

In situ hybridization

To make hybridization probes, the total RNA from each blastodermal stage was reverse transcribed, and the cDNA was amplified using the primers of chicken *NANOG*, *POUV*, and *EOMES* (Table 1). The PCR products of the correct size were cloned with the pGEM-T Easy Vector System (Promega, Madison, WI, USA). After sequence verification, the recombinant plasmids containing the genes of interest were amplified with T7 (5'-TGTAATACGACTCACTATAGGG-3') and SP6 (5'-CTATTTAG-GTGACACTATAGAAT-3') specific primers to prepare the templates for labeling with hybridization probes (Rengaraj *et al.*, 2008). Digoxigenin (DIG)-labeled sense and antisense hybridization probes of each gene were transcribed *in vitro* using the DIG RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland). Whole mount *in situ* hybridization was performed following the standard published protocol for chickens (Stern, 1998). In addition, intrauterine embryos were embedded in paraffin and sectioned at 10 μm on a HM 355S automatic microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After deparaffinization, rehydration, and antigen retrieval, each slide was mounted with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The embryonic nuclei were evaluated under a Ti-U fluorescence microscope (Nikon, Tokyo, Japan).

Measurement of cellular diameter

Longitudinal sections of intrauterine embryos were mounted with DAPI. Upper-most layer cells and detached lower layer cells from germinal disc into subgerminal cavity were observed using Ti-U fluorescence microscope. The diameter of each cell was measured across the center of nuclei using NIS-elements 3.0 imaging software (Nikon). The significance between two groups of cell type was examined statistically using Student's t-test. A P value < 0.05 was considered to indicate statistical significance.

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