

Screening of novel uterine proteins involved in eggshell formation using cDNA microarrays

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Summary

The chicken eggshell is a biomineral which protects the embryo during its development. Eggshell is formed in the uterus and its remarkable mechanical properties are due to the interaction between mineral and organic matrix. Genes coding proteins potentially involved in the eggshell formation were analysed using cDNA microarrays. The expression of genes in uterus was compared with two others regions of the oviduct (isthmus and magnum) and allowed the identification of 605 differentially expressed genes. Among these, 55 genes code novel proteins not yet characterized. The others genes were functionally annotated and many of them showed properties suggesting a putative role in eggshell formation were related to calcification process. Fifty four proteins were identified as secreted in uterine fluid to control biomineralisation of eggshell and classified according their biological relevance. Fourteen of them are able to bind ions and consequently could be implied in eggshell fabric. A potential antimicrobial role was also reported for 14 of them. The presence of negative charge at uterine pH and putative ion binding for Ca lattice were explored by studying isoelectric point (pI). Twenty nine proteins were identified with acidic theoretical pI and might interfere with calcium carbonate crystal. This study allowed the identification of a restricted group of genes coding proteins that could be related to the natural defences of egg.

Keywords: eggshell, mineralization, antimicrobial, microarrays

Introduction

The avian egg is a reproductive structure that has been shaped through evolution to resist physical, microbial and thermal attack from an external and possibly aggressive environment. It contains all the components that are required for a proper embryonic development: nutrients essential for embryogenesis, but also many molecules that participate in the growth and defence of the embryo. Natural egg defences consist of a physical barrier assured by the eggshell and membranes, and by chemical defence that encompass all the antimicrobial proteins which are distributed within the entire egg. Any disruption of eggshell properties and antimicrobial activities might directly affect the risk of egg contamination and of food-borne outbreaks for the consumer. Moreover, by 2012 the EU directive 1999/74 defining minimum standards for the protection of laying hens plans to abolish the conventional cage system in favour of furnished cages, aviaries or other floor systems in order to improve the welfare of the hens. These modifications in the housing systems might increase the microbial risk in eggs produced for human consumption. In such a context, the identification of new molecules involved in the physical and chemical defence of eggshell, and their characterization to explore the phenotypic and genotypic variability of both systems are of great importance to improve the eggshell's natural defences using marker assisted selection.

Egg proteins were classically studied using biochemical and molecular biology techniques. Despite these efforts, the composition of the egg is still not completely understood. Only the major proteins have been identified, and many minor egg proteins are not yet characterised. The recent development of high-throughput methods used in combination with the newly available chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004) yields new tools for the characterization of new and minor egg components. Egg proteins are specifically and sequentially expressed throughout the length of the oviduct. Magnum is the region of the oviduct where egg white proteins are synthesized and secreted to surround the yolk. White isthmus and uterus are the location of eggshell membranes formation and eggshell fabric respectively. In a recent study (Jonchere et al., 2009), we have used cDNA microarrays to identify genes over-expressed in the uterus in comparison with magnum and isthmus. This study allowed the identification and characterization of eggshell proteins potentially involved in transfer of precursors involved in eggshell formation.

Materials and methods

Unique clones from a reproductive tissue library (7,937) combined with the 9,833 unique cDNA from the metabolic/somatic library produced by the University of Delaware were used to produce the 14K DEL-MAR Chicken Integrated Systems Microarray (GEO Accession # GPL1731, Cogburn et al, 2004). Oviducal samples collected at different levels of this organ were used to determine the differentially expressed genes in the magnum, in the white isthmus and in the uterus. Hybridization experiments and statistical analysis were performed as described in Jonchere *et al.*, (2009). Briefly, total RNAs were extracted from frozen tissue samples. Twenty micrograms of total RNAs from various regions of oviduct were used to produce fluorescently labelled cDNA. The experimental design used a dye switch procedure in which half of samples were labelled with Alexa 555 fluorescent probe and the other half using Alexa 647. The uterine expression was first compared to magnum on a first set of 8 slides then to white isthmus on a second set of 8 slides. The GenePix Pro 6.0 software was used to acquire the fluorescent image, align the spots, quantify their intensity and finally export the GPR files containing spot intensity raw data. The profiling of differentially expressed genes was determined using Anapuce package (http://www.inapg.fr/ens_rech/math/outil.html) (Martin-Magnette *et Robin*, 2004). Assuming various group of variance, we estimated the gene variance using a mixture model implanted in varmixt method (Delmar *et al.* 2005). Taking account gene variance estimation, we performed unilateral statistical t-test. P-values were adjusted by a Benjamini-Hochberg (BH) multiple testing procedure, taking into account that all tests were simultaneous.

Results and discussion

A total of 2308 transcripts were over expressed in uterus when this tissue was compared to magnum (fold difference from 1.1 to 79.4). When uterus was compared to white isthmus, 718 transcripts were found to be over expressed in uterus (fold difference ranging from 1.1 to 21.8). Among the differentially expressed transcripts, a total of 605 transcripts were found to be simultaneously over expressed in uterus when compared to magnum and white isthmus. They correspond to the uterine transcripts coding specific proteins expressed during the eggshell calcification process (Jonchere *et al.*, 2009). The eggshell is made of calcium carbonate and a matrix playing an important role in the control of the calcification and the determination of its mechanical properties (Nys *et al.*, 2004, Gautron *et Nys*, 2007a). The organic matrix is made of numerous specific proteins including ovocleidins and ovocalyxins (Nys *et al.*, 2004; Gautron *et Nys*, 2007b). All these sequences corresponding to eggshell matrix proteins specifically expressed in

uterus were present on the cDNA array used in this study. They were found to be over expressed in the uterus. This observation validated the use of transcriptomic approach to identify genes coding eggshell proteins or involved in providing inorganic precursors (Jonchere *et al.*, 2009). The 605 uterine specific genes were found to correspond to 469 different genes identified in *Gallus gallus* (UniGene database) and to 437 different proteins identified in *Gallus gallus* (90 proteins), and by homology in human (161 proteins), in other mammals (163 proteins) and from various species (23 proteins). Additionally, 55 uterine specific genes were found to be proteins with no correspondence in public database suggesting that these genes encoded proteins which might be novel and specific of the eggshell. The software EASE (<http://david.abcc.ncifcrf.gov/ease/ease.jsp>) was used to obtain functional Gene Ontology (GO) terms for each gene and provided statistical methods for discovering enriched biological terms within the gene list. Significant terms with an EASE score greater than $p < 0.005$ were selected. The 469 over expressed genes in uterus were classified in families presenting the same potential functionality using tool EASE. These data suggested an important control of uterus activity by nervous system; an intense transcriptional activity which is related to an intense synthesis of proteins, the addition of acidic groups and saccharidic chain. It is also notable that a large group is constituted of proteins with an ability to transport and /or to bind ions and to regulate transport of protons.

Amongst these proteins, an attention was paid on the nucleotide sequences coding secreted proteins. The eggshell matrix proteins playing a crucial role in the mineralization process are initially secreted by uterine tissue before being incorporated in the calcified shell. For identifying secreted proteins, the 437 protein sequences were analysed using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) to determine the presence of the peptide leader sequence as this peptide is a prerequisite for a protein to be secreted. 12% of the genes over expressed in the uterus encoded for 54 secreted proteins. Their functions were examined using different databases and publications in the aim to classify proteins in various groups regarding their biological functions. The first group was related to proteins that could be involved in the biomineralization of the shell and its resulting mechanical properties. A number of experimental observations support the role of the eggshell matrix proteins in the fabric of the eggshell and its resulting mechanical properties (Nys *et al.*, 1999, Gautron *et Nys*, 2007a). In such a context, it is interesting to observe amongst the 54 secreted proteins, the presence of 10 calcium-binding proteins that were not yet characterized in the eggshell. These proteins are promising candidates for being involved in the eggshell calcification. We also paid particular attention to 2 already identified proteins known for their role in the mineralisation of others tissues (Dentin Matrix protein 4, and Mannose-binding protein C).

The second group of uterine secreted proteins that could play an active role in the shell is proteins with antimicrobial properties. Antibacterial activities have already been reported in the eggshell matrix (Mine *et al.*, 2003). Ovocalyxin-36 a specific eggshell matrix protein related to bactericidal permeability increasing protein might be partly responsible for this activity (Gautron *et al.*, 2007c). This transcript is 23 fold over expressed in uterus compared to magnum. AvBD-9 (Gallinacine-9), a cationic antimicrobial peptide, was also found over expressed in uterus. The study also reported new proteins as good candidates to an antimicrobial activity. It is notably observed for 3 proteins containing immunoglobulin-like domains. These molecules could have a similar role of immunoglobulins which are crucial in the interactions between the cells implied in the immune system. In parallel, we revealed 9 proteases or anti proteases which could limit the pathogenicity of microbes by inhibiting bacterial protease or by liberation of antimicrobial peptides from major proteins. Proteases, anti proteases and chaperone molecules also identified in the secreted proteins could also be involved in modulating activity of proteins in the uterine fluid which is the acellular milieu where the eggshell is calcified.

The matrix proteins can interact with the mineral phase by their negative charges that allow electrostatic interaction with calcium ions. Proteins with a low P_i relative to the pH of the uterine fluid (7.2-7.6) are likely candidates (Hernández-Hernández *et al.*, 2007; Marin *et al.*, 2008). These binding capacity with ions, influence the growth of crystals and their morphologies, numbers and sizes. *In fine*, the texture of the shell and their resulting mechanical properties are directly related to the interaction of mineral with eggshell matrix proteins. In order to identify these potential interaction sites, theoretical isoelectric point (p_i) and amino-acids composition were studied on the 54 secreted proteins. The study revealed 29 proteins with p_i lower than 6. Among these, osteopontin a well known protein to be implied in biomineralisation have been identified as very acid ($p_i=4.5$) and with a high concentration of glutamate (%E: 9.3) and aspartate (%D: 12.9). A total of 29 proteins acidic proteins were identified. They might bind calcium and are putative actors of the biomineralisation process.

Conclusion

This transcriptomic approach identified novel genes and proteins specifically expressed in oviduct regions where eggshell are formed. Complementary experimental studies should be necessary to confirm their biological activity. Then a selection of these new components will be analysed for their interest as biological markers the single polymorphisms of which can be used in marker assisted genetic selection (MAS) to improve eggshell strength and, consequently, to reduce the risk of food-borne disease outbreaks for consumers.

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