

Generation of novel hypothesis on the control of shell gland function from micro-array experiments; implications for shell quality

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Introduction: The oviduct of domestic hen grows and regresses with the activity of the reproductive system. The change in mass stimulated by ovarian steroids is around 30 fold and is accompanied by a prodigious capacity to secrete the components of the egg, including the shell. The eggshell forms in the shell gland region of the hen's oviduct over about 20 hours and is a highly ordered bio-ceramic of fused calcite (CaCO₃) crystal pillars formed on a protein skeleton with distinct layers and regular pores which allows gas exchange for the developing embryo. If the formation of the eggshell is compromised then an egg can suffer damage during handling and allow food spoilage organisms to enter the egg. Eggshell quality is therefore a priority for producers, retailers and consumers.

Objectives: We aimed to increase our understanding of the molecular mechanisms by which the shell gland forms eggshells using data derived from microarray experiments. In particular to identify the factors which may be important for the maintenance of a fully active shell gland and which may influence quality as the hen ages. To do this we compared samples taken from shell glands in sexually immature hens and from those that were sexually mature.

Experimental methods:

Animals, and sampling: Samples of shell gland tissue to make RNA samples were taken at two points in the laying cycle, pre lay and peak of lay (12 and 28 weeks of age), with 16 samples at each age. The samples from the birds at peak of lay were taken when there was no egg in the shell gland. The samples were taken from 8 hens of a high and 8 low selection line for bone quality at each sampling point. There was no significant difference in the two lines for body weight or egg production.

Microarray design: To ensure the microarray was appropriate for tissues involved in mineralisation such as the shell gland and bone, cDNA libraries were produced using suppressive subtraction hybridisation (SSH) PCR (Clontech) of both these tissues. The libraries were sequence sampled on two occasions to identify and remove clones that were over represented in the libraries. About 31% of the genes were novel. These genes were supplemented with candidate genes involved in egg shell formation, osteoporosis and bone mineralization from the BBSRC EST collection (Boardman et al., 2002) and clones derived from in-silico subtraction from the BBSRC chondrocyte EST library. The final composition of the micro-array was therefore as follows: 1) Candidate genes, 385 cDNAs; 2) Unique EST in Chondrocytes, 1543 cDNAs; 3) Clones from SSH Bone library, 2521 cDNAs; 4) Clones from SSH Shell Gland library, 2521 cDNAs plus 1000 random clones for normalisation of the array data.

RNA isolation, and microarray hybridization and analysis: Total RNA was isolated using Ultraspec II RNA (AMS Bioscience) and the quality checked before labelling using an Agilent bioanalyzer. The samples were hybridised in 16 randomised pairs at each age in a dye-swap design using Cy3 dye and Cy5 dye, labelling was performed using a Stratagene Fairplay™ kit and hybridised using an automated GeneTAC hybridisation station from Genomic Solutions.

The slides were scanned with a ScanArray 5000 and images were processed using Quantarray with manual checking of alignments and quality. Log mean intensity (A) values were analysed after quantile normalisation of cDNAs: superimposing the distribution for each chip onto an "average" distribution. Analysis for the age effect was performed using the between pairs stratum of a split plot ANOVA on the normalised A values.

Real-time quantitative reverse transcription (RT)- QPCR: RNA isolated as described above was reverse transcribed for use in real time PCR assays. A 4µl sample containing 1ug of total RNA was reverse transcribed using a First Strand synthesis kit (Amersham Pharmacia Biotech UK Ltd., Little Chalfont,

Buckinghamshire, UK). Reverse transcribed samples were diluted prior to use 20 fold with dH₂O. Primers to a selection of identified genes OPG, RAC2, SLC14a2, CAVIII and Thrombospondin, were designed using primer software. QPCR was carried out using 5ul of the diluted cDNA according to Brilliant Sybr green master mix (Stratagene) instructions with gene specific primers (0.6pM) . GAPDH was used as a control. PCR reactions were run on an ABI7700 Sequence Detection System (Applied Biosystems) using the following conditions 95C for 10min, then 40 cycles of (95C for 15 s, 60C for 1min). Controls (no template) were run for all primer pairs. Template dilution was used to confirm detection of differences in expression. Agarose gels were run to confirm that single bands were amplified by each primer pair using the conditions and reagents used in the assay. Eight replicates from juvenile and mature shell gland were used for confirmatory QPCR. Ct values were analysed using GAPDH Ct values as a covariant.

Results and discussion: We used quantile normalisation of microarrays of juvenile (12 weeks of age) and sexually mature (24 weeks of age) hens to utilise data gathered in experiments comparing genetic lines of white leghorns. This produced a list of genes with expressions levels that were up or down regulated in the mature shell gland compared to the juvenile and which did not overlap. In a list of 300 potentially differentially expressed genes false discovery rates were smaller than $1/1 \times 10^{-10}$. Real time PCR confirmed the differential expression of all genes selected for test both up regulated and down regulated in the mature shell gland. As expected a number of genes which encoded for proteins that are known to be part of the eggshell (Nys et al., 2004) such as ovocalyxin-32, ovocleidin-116, ovocalyxin-22, ovocalyxin-36 or part of the CaCO₃ secretory pathway such as carbonic anhydrase II and calbindin were present in the list. There were, however, many other genes we would not have predicted; amongst these were osteoprotegerin (OPG) which was up regulated in the mature shell gland. We have established hypothesis on how this gene may maintain the shell gland in an active state and may act to control calcium homeostasis and supply of calcium from bone. Osteoprotegerin is a decoy death receptor which modulates the differentiation of bone reabsorbing cells, the osteoclasts by neutralising RANKL (receptor activator of nuclear factor (NF- κ B) ligand) (Rogers and Eastell, 2005). We have hypothesised that the shell gland is a source of circulating osteoprotegerin which has an endocrine effect on mobilisation of calcium from bone by osteoclasts. Osteoprotegerin also prevents apoptosis by its ability to bind TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) to prevent its interaction with death receptors (Emery et al., 1998), and so we have further hypothesised that osteoprotegerin prevents apoptosis in the shell gland when the reproductive system is active. In addition to confirming differential expression by RT-QPCR support of the hypothesis derived from microarrays results comes from western blotting that confirmed osteoprotegerin protein is present in the shell gland. We have also established that TRAIL, one of osteoprotegerins targets is expressed in the shell gland. A related decoy death receptor, DcR3, was checked as a control for differential expression. This gene was not differentially expressed suggesting the effect on osteoprotegerin is specific.

In conclusion we have been able to derive new hypothesis on the function of the shell gland using results from microarrays which when fully tested will lead to a greater understanding of shell gland function and how a quality eggshell is formed.

- BOARDMAN, P. E., SANZ-EZQUERRO, J., OVERTON, I. M., BURT, D. W., BOSCH, E., FONG, W. T., TICKLE, C., BROWN, W. R. A., WILSON, S. A. & HUBBARD, S. J. (2002) A comprehensive collection of chicken cDNAs. *Current Biology*, 12, 1965-1969.
- EMERY, J. G., MCDONNELL, P., BURKE, M. B., DEEN, K. C., LYN, S., SILVERMAN, C., DUL, E., APPELBAUM, E. R., EICHMAN, C., DIPRINZIO, R., DODDS, R. A., JAMES, I. E., ROSENBERG, M., LEE, J. C. & YOUNG, P. R. (1998) Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *Journal of Biological Chemistry*, 273, 14363-14367.
- NYS, Y., GAUTRON, J., GARCIA-RUIZ, J. M. & HINCKE, M. T. (2004) Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *Comptes Rendus Palevol*, 3, 549-562.
- ROGERS, A. & EASTELL, R. (2005) REVIEW: Circulating osteoprotegerin and receptor activator for nuclear factor kappa B ligand: Clinical utility in metabolic bone disease assessment. *Journal of Clinical Endocrinology and Metabolism*, 90, 6323-6331.