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Extracellular Matrix Molecules of the Eggshell as Related to Eggshell Quality

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- ☐ **Cooperation**, briefly explain whether synergistic, complementary or supportive.

The US group established a library of monoclonal antibodies to eggshell gland proteins which are tested on eggshell gland biopsies taken by the IS group at different physiological stages. _____

b. Research Activities and Results

INTRODUCTION

The overall purpose of this research is to improve eggshell quality. As a first step toward this goal, it is necessary to discern the molecular constituents involved in the mineralization of the eggshell since they guide the mineralization processes. The primary goals for the laboratories at Case Western Reserve University and Volcani Center were to identify eggshell matrix proteins and to try to elucidate the regulation of these genes in response to eggshell formation. Few approaches were used:

- A- We generate a bank of monoclonal antibodies specific to eggshell matrix proteins. These antibodies will be used as tools for isolate and characterize eggshell matrix proteins. In the first part, we report the generation of such monoclonal antibodies that bound to demineralized eggshell matrix or localized to oviduct epithelium.
- B- We investigated the involvement of specific matrix proteins in eggshell formation. To do so, we evaluate the role of a battery of proteins known to participate in other calcification process (such as bone) in the eggshell formation. These include osteopontin, osteonectin, osteocalcin, dermatan sulfate and bone sialoprotein. We found that from this list only osteopontin and dermatan sulfate were expressed by the ESG. Only osteopontin was expressed in a circadian fashion.
- C- Using RNA fingerprinting technique we screened for genes that are differentially displayed when the egg enters the eggshell gland (ESG). We found genes that are known chicken genes, genes with high homology to mammalian genes and few new genes. The regulation of the expression of some of these genes was studied. Two main regulatory processes were found which affect the level of expression of these genes. The first is the calcium flux and the second is mechanical stress.
- D- We studied the regulation of the Calcium-sensing receptor in the laying hen. This receptor is responsible for the minute-to-minute regulation of the plasma calcium homeostasis by regulating the secretion of the parathyroid hormone from the parathyroid gland.

It is expected that the results obtained will eventually allow a correlation between the levels of particular molecular components in an eggshell and the eggshell mechanical strength. This information is of utmost importance for future planning strategies to improve hatchability and reduce eggshell cracking. The information can be used by the geneticist to select for better eggshell quality and can be incorporated into simulation algorithms for profit maximization in egg-laying operations.

MONOCLONAL ANTIBODIES TO MINERALIZED MATRIX MOLECULES OF THE AVIAN EGGSHELL

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ABSTRACT

The extracellular matrix of the mineralizing eggshell contains molecules hypothesized to be regulators of biomineralization. To study eggshell matrix molecules, a bank of monoclonal antibodies was generated that bound demineralized eggshell matrix or localized to oviduct epithelium. Immunofluorescence staining revealed several staining patterns for antibodies that recognized secretory cells: staining for a majority of columnar lining cells, staining for a minor sub-set of columnar lining cells, intensified staining within epithelial crypts, and staining of the entire tubular gland. Western blotting with the antibody Epi2 on eggshell matrix showed binding to molecules with the apparent molecular weight of eggshell matrix dermatan sulfate proteoglycan (eggshell DSPG) (Carrino, *et al.*, 1997). Immunoblots of cyanogen bromide-cleaved eggshell DSPG revealed a broad band of reactivity that shifted to 25 kDa after chondroitinase digestion; indicating that the Epi2 binding site is located on a fragment which contains dermatan sulfate side chains. Immunogold labeling showed that Epi2 binds to secretory vesicles within the non-ciliated cells of the columnar epithelium, while the antibodies Tg1 and Tg2 bind to secretory vesicles of tubular gland cells. Immunogold labeling of demineralized shell matrix showed binding of Epi2, Tg1, and Tg2 to the matrix of the palisades layer, and showed little reactivity to other regions of the shell matrix. Quantification of the immunogold particles within the eggshell matrix revealed that antibodies Epi2 and Tg1 bind all calcified regions equally while antibody Tg2 has a greater affinity for the baseplate region of the calcium reserve assembly.

INTRODUCTION

The avian eggshell is useful as a model system for studying biologic regulation of the

mineralization process. The components of the eggshell are sequentially assembled onto the egg surface as it transits the oviduct. A single egg of a chicken (*Gallus gallus*) forms approximately 5 g of calcium carbonate within 20-24 hr, which makes this one of the most rapid mineralization events in nature (Simkiss, 1961). Most of the matrix material is delivered to the surface of the egg by columnar epithelial cells that line the oviduct and by tubular gland cells via ducts leading to the oviduct lumen (Wyburn, *et al.*, 1970, Draper, *et al.*, 1972). The innermost region of the eggshell matrix consists of a pair of non-mineralized matrix layers composed of highly cross-linked type I and type V collagens arranged in thick fibers; these matrix layers are termed the shell membranes. The surfaces of individual shell membrane fibers are coated with a highly glycosylated mantle (Simkiss, *et al.*, 1957, Baker, *et al.*, 1962, Arias, *et al.*, 1992, Dennis, *et al.*, 1996), and the cores have been shown to contain type X collagen (Arias, *et al.*, 1991, Arias, *et al.*, 1997). The shell membranes are in a non-mineralizing region of the shell matrix. The mineralized region of the matrix is morphologically complex and is thought to contain areas having specialized functions with respect to mineralization. For example, the calcium reserve assembly (CRA) is named for its function as the site within the eggshell that is selectively dissolved for use by the developing embryo; it is Areserved@ for the embryo (Dieckert, *et al.*, 1989). The CRA is assembled while the egg resides within the isthmus of the oviduct (Wyburn, *et al.*, 1970, Creger, *et al.*, 1976, Stemberger, *et al.*, 1977), and the CRA contains sites at which calcite crystals are first formed (Dieckert, *et al.*, 1989), which indicates that there may be calcite initiation sites within the CRA matrix. The crown and palisade regions have a matrix structure that is distinct from that of the calcium reserve assembly (Dennis, *et al.*, 1996), and these regions are where calcite crystals assume a preferred crystallographic orientation (Perrott, *et al.*, 1981, Silyn-Roberts, *et al.*, 1989), and is also the region of most rapid crystal growth. The palisade region is capped by a cuticle which is suspected to contain calcite growth inhibitory molecules (Nys, *et al.*, 1991). The prominent cuticle structures are cuticle vesicles, 200-500 nm spheres, which contain hydroxyapatite

needles in the vesicles proximal to the palisade region (Dennis, *et al.*, 1996).

Few molecules of the eggshell matrix have been described in detail. The non-mineralizing shell membrane region contains collagen types I, V (Wong, *et al.*, 1984) and X (Arias, *et al.*, 1991, Arias, *et al.*, 1997), and glycosaminoglycans (Simkiss, *et al.*, 1957, Baker, *et al.*, 1962, Arias, *et al.*, 1992). The molecules identified in the mineralized matrix include osteopontin (Pines, *et al.*, 1995), ovocleidin 17 (Hincke, *et al.*, 1995), ovalbumin (Hincke, 1995), and a dermatan sulfate proteoglycan (Carrino, *et al.*, 1997). There is evidence that the mineralized region also contains keratan sulfate proteoglycans (Arias, *et al.*, 1992, Fernandez, *et al.*, 1997), although there is no report on the isolation of a specific keratan sulfate-containing molecule.

In order to further characterize the matrix molecules of the avian eggshell matrix, a bank of monoclonal antibodies was prepared from mice immunized with demineralized avian shell matrix. Hybridomas were selected based on their ability to produce antibodies that stained positively for eggshell matrix on ELISA and also either stained positively on immunoblots of demineralized eggshell matrix or stained the secretory cells of the shell gland by immunofluorescence. The immunostaining data revealed specific binding of antibodies to different epithelial cells of the shell gland. Electron microscopic localization confirmed the binding of antibody to secretory granules and showed preferential binding of one antibody, Tg2, to the baseplate region of the shell matrix. Another antibody, Epi2, was shown to bind an eggshell dermatan sulfate proteoglycan which was previously described in this laboratory.

RESULTS

Monoclonal Antibody Screening

A total of 1,920 wells of hybridoma cells were tested; of these, 1,554 showed colony growth and 229 wells were positive on ELISAs against immunogen (demineralized eggshell

matrix). Of these, 60 clones showed positive immunofluorescent staining on frozen sections of shell gland and 16 showed reactivity on immunoblots of shell matrix extract. A total of 13 hybridomas were expanded and showed stable expression of antibody, while an additional 30 hybridomas were frozen for cloning at a later date. Samples saved for later showed staining patterns similar to hybridomas already selected for cloning and expansion, but may turn out to be specific for other molecules.

Immunofluorescent staining of the shell gland was used to select hybridomas that were likely to produce antibodies against secreted components of the eggshell matrix. The most common patterns of staining for the different antibodies is shown in Figure 1. The columnar epithelium that directly lines the lumen of the oviduct showed three distinct staining patterns which are localized to sub-sets of the columnar epithelial cells: 1. Staining of a majority of the epithelial cells, 2. Staining predominantly localized to crypts interpreted to be ducts arising from the tubular gland, and 3. Staining of a small subset of epithelial cells. One of these antibodies, Epi2, intensely stained a majority of the columnar cells lining the oviduct (Figure 1A), while another lining cell-specific antibody, Epi1, is an example of an antibody that labeled the crypts of the columnar epithelium (Figure 1B). An example of an antibody that binds a minority of the columnar epithelial cells is shown in Figure 1C. The tubular gland secretory cells were generally homogeneous in their reactivity with the monoclonal antibodies, most being positive for all secretory cells within the tubular gland (Figure 1E). The single exception was antibody ESS1 that showed positive staining in vesicles within approximately 50% of the tubular gland cells (Figure 1E). Figure 1F shows the staining pattern for a monoclonal antibody (ECM1; extracellularmatrix 1) that stained both the apical region of the columnar epithelium and the extracellular matrix throughout the shell gland.

Many of the antibodies that tested positive for the immunogen on ELISA were rejected based on their staining pattern on shell gland sections. For example, at least 4 hybridomas were found to stain the nuclei of all cells, and a number of antibodies were found to stain the

extracellularmatrix of the shell gland, such as ECM1 in Figure 1F. These antigens are clearly not of interest with respect to matrix molecules that may regulate mineralization and probably represent antigenic molecules that became imbedded in the mineralized shell matrix as debris. Indeed, a close examination of the antibody staining pattern in Figure 1D shows that although this antibody is specific for an epithelium cell, the intracellular staining pattern is more reminiscent of that of a filamentous structure than that of a secretory protein.

Tissue Distribution

To characterize the specificity of staining, selected antibodies (Tg1, Epi2, Epi4, Epi5, and Epi6) were tested for their affinity to tissues from an adult chicken (shell gland, liver, kidney, muscle, trachea, cartilage, and small intestine). Table I shows the reactivity of each of these antibodies to the different tissues, and examples of the observed staining patterns are shown in figure 2. There were a few antibodies that reacted to the epithelium of trachea (Figure 2 A-F), and one antibody, Epi5, reacted to the nuclei of liver and kidney cells (2 G-J), but not to nuclei in the other tissues, such as muscle (2 K, L). Confocal microscopy of Epi5 staining within the kidney nuclei (2J) showed Epi5 staining that did not overlap with propidium iodine staining of DNA, as no yellow color (indicating red and green overlap) was detected. A strong and distinctive staining pattern for Epi4 was observed on sections of kidney, wherein Epi4 strongly stained distal kidney tubules while proximal tubules and glomeruli were completely negative (Fig 2M, N). As detailed in Table I, there was no staining detected for any of the antibodies on frozen sections of muscle, small intestine, heart, lung, or incartilage.

Epi2 Antibody Characterization

One antibody, Epi2, was shown on immunoblots to recognize a diffuse band of the

same molecular weight as eggshell DSPG (Carrino, *et al.*, 1997). Previous results showed that eggshell DSPG is recognized by the monoclonal antibody MY174, which was shown to bind to the core protein of the proteoglycan PG-M (or versican; Yamagata *et al.*, 1993). Western blots of shell gland extracts and purified versican showed that Epi2 bound to 3 prominent high molecular weight bands (greater than 300 kDa), and, in chondroitinase ABC-digested samples, a set of smaller bands at approximately 200, 140 and 68 kDa (Figure 3). Epi2 antibody did not bind to any bands from purified versican on western blots while MY-174, which also recognizes eggshell DSPG, showed positive bands on versican blots, as previously reported (Yamagata, *et al.*, 1993, Carrino, *et al.*, 1994). These data indicate that Epi2 and MY-174 both recognize eggshell DSPG, but that Epi2 is more specific for eggshell DSPG than MY-174 in that it does not cross-react with versican.

To further characterize the epitope for Epi2, purified eggshell DSPG and cyanogen bromide-cleaved eggshell DSPG, with and without chondroitinase and keratanase digestion, were probed with Epi2 antibody on western blots (Figure 4). Digestion of the whole molecule with chondroitinase removed the broad band of staining above around 150 kDa, as described earlier (Carrino, *et al.*, 1994). Cyanogen bromide fragments without further digestion contained only traces of diffuse staining at low molecular weights. Upon digestion with chondroitinase ABC, more prominent bands appeared at around 25 kDa. Further treatment with keratanase did not change the migration distance of the prominent cyanogen-bromide protein fragments. These results indicate that the epitope for Epi2 is a 25 kDa-fragment, which contains dermatan sulfate side chains, and little or no keratan sulfate.

Immunogold Localization

Selected monoclonal antibodies were incubated with ultra-thin sections of LR White-embedded samples of shell gland and demineralized eggshell matrix and visualized by electron microscopy with the use of gold-labeled secondary antibody. Immunogold localization of Epi2

on shell gland sections demonstrated that the antigen for Epi2 is localized within secretory granules (Figure 5A) in the columnar epithelium of the shell gland. The results also showed that Epi2 is localized to a sub-set of secretory columnar epithelial cells, as some neighboring cells containing secretory granules contained no gold-positive granules. The monoclonal antibodies Tg1 (Figure 5B) and Tg2 (Figure 5C) stained secretory granules within the tubular gland cells and were negative on columnar epithelial cells. Tg1 stained secretory granules and also stained peroxisomes, which indicates that the antigen for Tg1 is probably at least partially recycled through the peroxisomes and that the Tg1 epitope is not completely lost during this recycling. The Tg2 antigen was observed within secretory granules at a higher staining density than Tg1, which indicates either a greater antigen affinity or greater number of epitopes for Tg2 compared to Tg1. Only background staining for Tg2 is observed in peroxisomes, indicating either the loss of this epitope within the peroxisomes or that the Tg2 antigen is not recycled.

In the demineralized eggshell matrix, antibodies Epi2 and Tg1 were preferentially localized to the matrix and vesicles within the palisades region (Figure 5D and 5E). Antibody Tg2 was also localized to the palisade region and also showed preferential binding to the matrix located at the attachment site for the calcium reserve assembly and the shell membranes (Figure 5F), the region termed the baseplate (Dieckert, *et al.*, 1989). To test this observation, the density of gold particles was quantified for each of the antibodies for the baseplate and palisades regions of the demineralized eggshell matrix (Table II). These results confirmed that Tg2 contained a higher concentration of gold particles in the baseplate region than in other regions of the eggshell matrix. The antibodies Tg1 and Epi2 showed a generalized staining throughout the palisade region which was above background, and showed no particular affinity for any sub-regions within the baseplate, crown, or palisade regions. These results show that antibodies Epi2, Tg1, and Tg2 recognize secreted antigens from either the columnar epithelium (Epi2) or the tubular gland (Tg1 and Tg2) that become localized in the calcifying region of the eggshell matrix, and that Tg2 has a preferred affinity for the baseplate, the region where the

calcium reserve assembly attaches to the shell membranes.

DISCUSSION

The eggshell matrix of the chicken (*Gallus gallus*) is comprised of a structurally complex arrangement of matrix molecules (Dennis, *et al.*, 1996). In addition, the mineral component of the eggshell is not structurally homogeneous (Silyn-Roberts, *et al.*, 1989), and many of different properties of the mineral at different locations within the eggshell coincide with unique matrix structures, such as the dense fibrillar network associated with the calcium reserve assembly (Dieckert, *et al.*, 1989) and the hydroxyapatite needles embedded within cuticle vesicles (Dennis, *et al.*, 1996). It is hypothesized that the matrix imparts or directs the formation of these unique mineral structures within the eggshell, such as the initiation sites for crystal formation, the inhibition of calcite growth at the cuticle-palisade interface, the hydroxyapatite crystals within cuticle vesicles, and the region of preferential calcite dissolution during mineral mobilization by the developing embryo. The goal of this study was to generate a bank of monoclonal antibodies to eggshell extracellular matrix to use as tools for dissecting and identifying the mechanism of matrix and mineral assembly and determining the functions of the underlying eggshell matrix.

The eggshell is mineralized in a specialized region of the oviduct, the shell gland, which is responsible for transporting the matrix and minerals needed to calcify the shell and terminate the mineralization process. To date, only a few of the molecules within the calcifying matrix have been identified: osteopontin (Pines, *et al.*, 1995), ovocleiden 17 (Hincke, *et al.*, 1995, Mann, *et al.*, 1999), ovalbumin (Hincke, 1995), and eggshell dermatan sulfate proteoglycan (Carrino, *et al.*, 1997). Efforts to identify matrix components by conventional biochemical methods are likely to be hampered by high levels of covalent cross-linking in shell matrix, which renders a large percentage of the material unextractable (approximately 50%, in our experience

[Carrino, *et al.*, 1997]). One approach to circumvent the extractability problem is to extract the material from oviductal fluid (Nys, *et al.*, 1989, Nys, *et al.*, 1991, Gautron, *et al.*, 1997). In the present study, monoclonal antibody probes were generated against demineralized eggshell matrix as a way of characterizing, or providing reagents to characterize both the soluble and insoluble components of the shell matrix. Screening of the monoclonal antibodies against the secretory cells of the chicken oviduct allows identification of antibodies that recognize precursor molecules of the shell matrix. Thus, all selected antibodies will recognize unassembled (non-cross-linked) antigens within shell matrix, which may or may not retain antigenicity after cross-linking. Since unassembled antigens will be recognized, shell gland cells can be used as a source of shell matrix material for biochemical purification.

Western blot analysis demonstrated that Epi2 recognizes eggshell DSPG without cross-reacting to with versican (PG-M) (Ito, *et al.*, 1995). This is in contract to MY-174, which was originally described as a PG-M-specific (Shinomura, *et al.*, 1990) antibody, but was found to cross-react with eggshell DSPG (Carrino, *et al.*, 1997). A screening of other adult chicken organs (cardiac and striated muscle, kidney, trachea, lung, bone, small intestine, and liver) showed that Epi2 also stains the apical region of epithelial cells lining the trachea. It has not been determined if Epi2 recognizes the same molecule in the trachea as it does in the oviduct. If Epi2 does recognize an identical core protein at these two sites, one could postulate that the Epi2-molecule is a secreted molecule commonly found in mucous, suggesting that the localization of eggshell DSPG is perfunctory. Previous results have shown that eggshell DSPG is capable of modifying calcite crystal growth and morphology *in vitro* (Wu, *et al.*, 1995), which supports the idea that eggshell DSPG is a regulator of the eggshell mineralization process. Therefore, even if the DSPG core protein is found in both eggshell matrix and in the trachea, structural differences in the glycosaminoglycan side chains could define the functional role of this molecule at these two different sites.

Electron microscopic examination of immunogold-labeled sections confirmed that the

immunofluorescence staining observed in the columnar epithelium and the tubular glands is localized to secretory granules. Ultrastructural localization of the shell gland matrix showed localization of antibodies Epi2, Tg1, and Tg2 to matrix structures. Only antibody Tg1 was shown to preferentially localize to a sub-region (the baseplate) within the calcitic portion of the eggshell. To date, only a few molecules have been localized to specific eggshell matrix structures by electron microscopic methods. Immunostaining for type X collagen was observed in the non-calcified regions of the shell membranes by light microscopic methods (Arias, *et al.*, 1991, Arias, *et al.*, 1997) and was recently shown, by electron microscopy, within vesicles of the tubular gland in the isthmus region of the oviduct (Fernandez, *et al.*, 1999). The presence of a keratan sulfate proteoglycan within the calcium reserve body has been indicated by immunofluorescence staining with antibodies (5D4 (Caterson, *et al.*, 1983), 4D1, 8C2, and 3D2 (Sorrell, *et al.*, 1990)) specific to keratan sulfate (Arias, *et al.*, 1992). Recently, keratan sulfate has been localized, ultrastructurally, to the tubular gland cells within the isthmus of the oviduct with the 5D4 (Mehmet, *et al.*, 1986) antibody to keratan sulfate proteoglycans (Fernandez, *et al.*, 1999). As yet, no protein core has been identified that corresponds to the keratan sulfate glycosaminoglycans. In the same study (Fernandez, *et al.*, 1999), dermatan sulfate glycosaminoglycans were localized to electron-dense vesicles of non-ciliated columnar epithelial cells of the shell gland, an observation that fits with the data presented here for the core protein-specific antibody Epi2. The dermatan sulfate proteoglycan of the shell matrix has been termed Aovoglycan@ (Fernandez, *et al.*, 1999). The complete mRNA sequence for eggshell dermatan sulfate proteoglycan (termed Aovocleidin-116") is known (Hincke, *et al.*, 1999), and contains a deduced amino acid sequence identical to the partial sequence reported for eggshell DSPG (Carrino, *et al.*, 1997). While the term ovoglycan fits the accepted nomenclature for proteoglycans and ovocleidin-116 is a consistent method of naming eggshell proteins, neither name descriptively accommodates the localization of this DSPG molecule to the epithelial cells of the trachea.

The antibodies described in this report have been shown to localize to the secretory cells of the mineralizing region of the chicken oviduct, and the antibody Epi2 has been shown to recognize the eggshell DSPG molecule in a more specific way than does MY-174. These antibodies will be used in the future to directly isolate molecules by affinity chromatography or will be used in assays to develop conventional isolation techniques. It is suspected that some of these molecules will directly or indirectly contribute to the mineralization of the avian eggshell, and a detailed examination of these molecules will contribute to our understanding of matrix-mineral interactions.

EXPERIMENTAL PROCEDURES

Monoclonal Antibody Production: Eggshells from domestic fowl (*Gallus gallus*) were demineralized in 1 N hydrochloric acid, dialyzed at 4°C against distilled water and lyophilized to dryness. Shell matrix extract was suspended in PBS (150 mM sodium chloride, 10 mM sodium phosphate, pH 7.2) at 1.5 mg/ml, mixed with an equal volume of complete Freund's adjuvant, and injected intra-peritoneally into C57Bl6 mice. Mice were boosted with the same amount of antigen mixed with incomplete Freund's adjuvant once per week for four weeks. One week following the last boost, one mouse was sacrificed and the splenocytes were harvested and fused (in polyethylene glycol) with myeloma cells by standard methods (Kohler, *et al.*, 1975). Cells were plated into 96-well plates and cultured in HAT (hypoxanthine, aminopterin, thymidine) selection medium. Production of antibodies to eggshell matrix was tested by reactivity to shell matrix extract in ELISAs. ELISA cells were coated with shell extract solubilized in 8M urea, 0.15 M sodium chloride, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 0.05 M sodium acetate, pH 7.0 at 5 µg of shell extract in 25 µl per well. ELISA-positive hybridomas were tested for immunofluorescent staining on frozen sections of chicken shell gland and were tested for reactivity on immunoblots of eggshell matrix extract.

Antibody Affinity Purification: Antibodies used for Ultrastructural localization were affinity purified on Sepharose-4B coupled to goat anti-mouse IgG (Organon Teknika, Durham, NC). Antibody supernatant was applied to the Sepharose-4B column, and the bound antibody was

washed extensively with PBS, eluted with 0.2 M glycine-HCl (pH 2.3) into a 1/5 volume of 1.0 M Tris (pH 8.6), and dialyzed against PBS.

Immunofluorescence: For immunofluorescent staining, shell gland tissue was cut into approximately 1-cm³ pieces and flash frozen in liquid nitrogen-cooled isopentane. Sections 5-6 μ m thick were stained as described previously (Arias, *et al.*, 1991, Fernandez, *et al.*, 1991, Lennon, *et al.*, 1991) and observed with an Olympus BH-2 fluorescent microscope; kidney sections were also observed in a Zeiss LSM-410 confocal microscope (Carl Zeiss, Inc., Thornwood, NY). Sections were incubated in undiluted hybridoma supernatant and the secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse IgG, IgM, and IgA (Sigma, Inc., St. Louis, MO).

Ultrastructural Localization: The calcified eggshell was broken into 50- to 100-mm² pieces, which were washed briefly in water, placed in 3,500 Dalton nominal molecular weight cut-off dialysis tubing in a minimal volume of EDTA-fixative consisting of 10% ethylenediamine tetraacetic acid (EDTA), pH 7.3, containing 0.05% glutaraldehyde, 2% paraformaldehyde. The dialysis tubing was then immersed in 50 ml of EDTA-fixative. After 8 hr at room temperature with stirring, the solution was exchanged with fresh fixative containing EDTA and incubated overnight. The following day, the fixative solution was exchanged and the sample incubated for an additional 3 hr. Shell gland samples were fixed in 0.1% glutaraldehyde plus 4 % paraformaldehyde in 80 mM sodium cacodylate, 0.05 % calcium chloride, and pH 7.3 for 1 hr at 4⁰ C. The fixed eggshell and shell gland samples were then dehydrated in a graded series of ethanol and embedded in L. R. White acrylic resin (Polysciences, Ft. Washington, PA). Ultrathin (70-90 nm) sections were collected on 400-mesh nickel grids. Affinity-purified Epi2 antibody and control non-immune mouse IgG were diluted in 0.1% bovine serum albumen (BSA) in TBS (150 mM sodium chloride, 10 mM tris (hydroxymethyl)aminomethane, pH 7.6) at antibody concentrations of 0, 5, 20, 50, 100, and 200 μ g/ml. The sections on nickel grids were first incubated in 1% BSA in TBS for 30 min, followed by primary antibody for 48 hr at 4⁰ C. The sections were then washed 3 times with TBS, 5 min each, and incubated overnight at 4⁰ C in 15-nm gold-conjugated goat anti-mouse IgG/IgM (Amersham Life Science, Arlington Heights, IL) which was diluted 1/25 into 0.1% BSA in TBS. The samples were washed 3 times with TBS, 5 min each, fixed for 5 min in 2.5% glutaraldehyde in PBS for 5 min, washed 3 times with distilled water, stained with lead citrate, and observed in a JEOL 100CXII electron microscope operated at 80 keV.

For gold particle density analysis, micrographs were overlaid with a grid, and the area of the different eggshell structures (palisades, crown, calcium reserve body [CRB], baseplate, and the fiber and mantel of the shell membranes) were derived by the point intercept method. The ultrastructure of the different eggshell matrix structures has been described in detail (Dennis, *et al.*, 1996). The density of gold particles for each structure is

expressed as gold particles per square micrometer. To facilitate comparison and to compensate for differences in the non-specific reactivity of the different antibodies, the particle density results were also normalized to the particle density observed within the fiber and mantle (normalized gold density = structure gold density / fiber + mantle gold density). The fiber and mantle structures were defined, *a posteriori*, as an internal standard for non-specific staining.

Western Blotting: Shell matrix proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reduced and non-reduced conditions, and electro-transferred to Immobilon-P as previously described (Carrino, *et al.*, 1997). Affinity purified Epi2 antibody was diluted to 1 μ g/ml in 5% non-fat dry milk in TBST (TBS plus 0.1% Tween-20), and was stained and visualized with alkaline phosphatase-conjugated secondary antibody (Promega Biotech, Madison, WI) by previously described methods (Dennis, *et al.*, 1990, Lennon, *et al.*, 1991).

Isolation and Treatment of Proteoglycans

Eggshell matrix dermatan sulfate proteoglycan was isolated from eggshell and versican from chick embryonic skeletal muscle by anion exchange chromatography as previously described (Carrino, *et al.*, 1994, Carrino, *et al.*, 1997). Digestion of proteoglycans with chondroitinase and/or keratanase in the presence of protease inhibitors was performed as described previously (Carrino, *et al.*, 1994, Carrino, *et al.*, 1997). Treatment of proteoglycans with cyanogen bromide was conducted as previously reported (Dennis, *et al.*, 1990).

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FIGURE LEGENDS

Figure 1: Immunofluorescent staining of frozen sections of shell gland. Epi2 antibody (A) stains approximately 50% of the cells within the pseudostratified columnar epithelium of the shell gland while the tubular gland is completely negative. The antibody Epi1 intensely stains the pseudostratified epithelium at specific foci (crypts) where ducts from the tubular gland lead to the oviduct lumen (B). Another columnar epithelium-specific antibody, Epi5, shows positive staining in only a small percentage of pseudostratified epithelial cells (C). (D) shows tubular gland-specific staining for the Tg1 hybridoma while (E) shows the only hybridoma (ESS1) which showed staining for a subset of tubular gland cells. The antibody ECM1 bound bound extracellular matrix and apical epithelium of the lining cells. The general structure of the pseudostratified epithelial cells and the tubular gland are evident in this micrograph. TG=tubular gland; PE=pseudostratified epithelium.

Figure 2: Immunolocalization of antibodies to organs and tissues. Trachia epithelium bound to antibodies Epi2 (A) and Epi4 (B); compare to negative control (C). Antibody Epi5 also binds to trachia epithelium (D), especially within crypts. Double exposure with DNA-binding propidium iodine (PI) shows the location of nuclei within the trachia and lamina propria (E); yellow areas indicate regions of red (PI) and green (Epi5) overlap; matching phase contrast image is shown in (F). Epi5 localization to spots within nuclei of liver cells is shown in sections visualized with FITC filter only (G) and in a double exposure of Epi5 and PI (H); negative control sections show some background staining to occasional droplets (I). Confocal microscopy (0.5 micron optical sectioning) of kidney cells stained with Epi5 and PI (J). Note the localization of Epi5 within nuclei with a lack of yellow staining, indicating that 15B4 and DNA are not co-localized. Lack of Epi5 staining in muscle nuclei (K); micrograph of PI staining in the same section (L). Epi4 localizes to tubules within the kidney (M), but no staining is detected in glomeruli (g) or proximal tubules. Arrows point to proximal tubules in the immunofluorescence and phase (N) micrographs.

Figure 3: Western blots of eggshell DSPG (DSPG), with and without chondroitinase ABC digestion (CSase) and versican stained with antibody to PG-M (MY-174(Ito, *et*

al., 1995)), and DSPG and versican stained with Epi2 antibody. MY-174 recognizes both DSPG and versican while Epi2 stains only DSPG.

Figure 4: Epi2 western blot of eggshell DSPG with and without chondroitinase ABC (CSase) or keratinase (KSase) digestion, or cyanogen bromide (CNBr) cleavage. Pluses and minuses indicate whether or not the sample has been Csase, Ksase digested or cleaved with CNBr. Epi2 recognizes undigested and chondroitinased eggshell DSPG, as shown earlier, but does not recognize any prominent bands on CNBr-cleaved samples except when the CNBr fragments are also digested with chondroitinase. Chondroitinase digested CNBr fragments co-digested with keratinase did not show any change in gel migration, indicating that little or no keratan sulfate is present.

Figure 5: Immunogold localization of Epi2 (A and D), Tg1 (B and E), and Tg2 (C and F) on sections of Shell Gland (A-C) and sections of demineralized eggshell matrix (D-F). Granules in the apical portion of a pseudostratified epithelial cell (A) are clearly labeled with 10 nm gold while dark granules of a neighboring cell are negative (asterisk). Arrows in B and C point to granules in a tubular gland cell labeled with gold particles; M=mitochondria; N=nucleus; L=lumen. D and E are from the palisades region of the eggshell, and arrows point to palisades vesicles. Immunogold labeling with Tg2 is most prominent in the baseplate (BP) region.

CSase	-	+	-	+	+
KSase	-	-	-	-	+
CNBr	-	-	+	+	+

Table I: Immunofluorescent staining of monoclonal antibodies on adult chicken organs.

Tissue	Monoclonal Antibody				
	T g l	E p i 2	E pi 6	Epi 5	Ep i4
Shell Gland	+	+	+	++	+
	+	+	+	+	+
	+	+	+		+
Trachea		+	+	+	+
	+	+	+		+
	+				
Liver	-	-	-	+	+
Kidney	-	-	-	++	-
Muscle	-	-	-	-	-
S.I. ¹	-	-	-	-	-
Heart	-	-	-	-	-
Lung	-	-	-	-	-
Cartilage	-	-	-	-	-

¹S.I. = small intestine; +++ = high intensity fluorescent signal;

++ = moderate intensity; + = low intensity; - = background.

List of Publications

Manuscripts

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- Lavelin, I., Meiri, N. and Pines, M. 1999 New insight in eggshell formation *Poultry Sci* 79, 1014-1017
- Yarden, N., Lavelin, I., Genina, O., Hurwitz, S., Diaz, R., Brown, E.M. and Pines M. 2000 Expression of calcium-sensing receptor gene by avian parathyroid gland *in vivo*: Relationship to plasma calcium. *Gen. Comp. Endocrinol.* 117, 173-181.
- Dennis, JE, Carrino, DA, Yamashita, K and Caplan, AI. Monoclonal antibodies to mineralized matrix molecules of the avian eggshell. Submitted.

Chapter in Books

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Symposia

- Lavelin, I, Bar, A. and Pines, M. Regulation of the osteopontin gene during eggshell formation. 10th European Poultry Conference. pp246-249, June 1998, Jerusalem, Israel
- Yarden, N., Hurwitz, S. and Pines, M. calcium receptor gene expression in the chicken parathyroid gland. 10th European Poultry Conference. pp284-287 June 1998, Jerusalem, Israel
- Lavelin, I, Meiri, N, and Pines, M. Molecular approach to identify proteins involved in eggshell formation. The World Poultry Science Association, The 37th Annual Convention, March 2000, Zichron Yaacov, Israel
- Lavelin, I, Meiri, N, and Pines, M. The use of RNA fingerprinting to identify proteins involved in eggshell formation: A role for Na-K-ATPase. XXI World's Poultry Congress, August 2000, Montreal, Canada