Cell. Mol. Life Sci. (2009) 66:2707–2719
DOI 10.1007/s00018-009-0046-y

Cellular and Molecular Life Sciences

REVIEW

Protein constituents of the eggshell: eggshell-specific matrix proteins

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Received: 26 March 2009 / Revised: 23 April 2009 / Accepted: 24 April 2009 / Published online: 19 May 2009

Abstract In this article, we review the results of recent proteomic and genomic analyses of eggshell matrix proteins and draw attention to the impact of these data on current understanding of eggshell formation and function. Eggshell-specific matrix proteins from avian (ovocleidins and ovocalyxins) and non-avian (paleovaterin) shells are discussed. Two possible roles for eggshell-specific matrix proteins have been proposed; both reflect the protective function of the eggshell in avian reproduction: regulation of eggshell mineralization and antimicrobial defense. An emerging concept is the dual role (mineralization/antimicrobial protection) that certain eggshell matrix proteins can play.

Keywords Eggshell · Ovocleidin · Ovocalyxin · Mineralization · Calcite · Antimicrobial protection

Introduction

The avian egg is a reproductive structure that has been shaped through evolution to resist physical, microbial and thermal challenges from the external environment, while satisfying the needs of the developing embryo. This complex structure regulates the exchange of metabolic gases and water, and provides calcium to the growing embryo. Many studies have been conducted on avian eggs and most have centered on the egg of the domestic chicken. This considerable body of work has provided insight into the function and structure of the eggshell. In this article, we will review the results of recent proteomic and genomic analyses of eggshell matrix proteins and draw attention to the impact of this data on current understanding of their role in eggshell formation and function. Comparisons with the recently available genome sequence for Zebra Finch (*Taeniopygia guttata*) are made where possible (http://www.ncbi.nlm.nih.gov/projects/genome/guide/finch/). Finally, information about the non-avian turtle eggshell is presented.

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Overview of eggshell biosynthesis

The egg is assembled as it passes through specialized regions of the oviduct. It is composed of a central yolk surrounded by the albumen, eggshell membranes, calcified eggshell and cuticle. Following ovulation, the yolk/ovum complex travels through the longest portion of the oviduct (magnum) and progressively acquires the water, ions and proteins that compose the albumen (egg white) during a 2- to 3-h period [1]. Next, the yolk and albumen complex traverse the isthmus. In the proximal (white) isthmus, the inner and outer shell membranes are deposited during a 1- to 2-h period as a highly cross-linked fibrous meshwork. These are considered to be the innermost layers of the eggshell. The inner membranes remain uncalcified, while the fibers of the outer shell membrane penetrate the mammillary cones of the calcified shell [2, 3]. The shell membranes contain type I, V and X collagens [4–6]. In the distal (red) isthmus, organic aggregates are deposited on the surface of the outer eggshell membranes in a quasi-periodic array. Calcium carbonate mineralization at these nucleation sites is the origin of the mammillary cones (Fig. 1) [3].

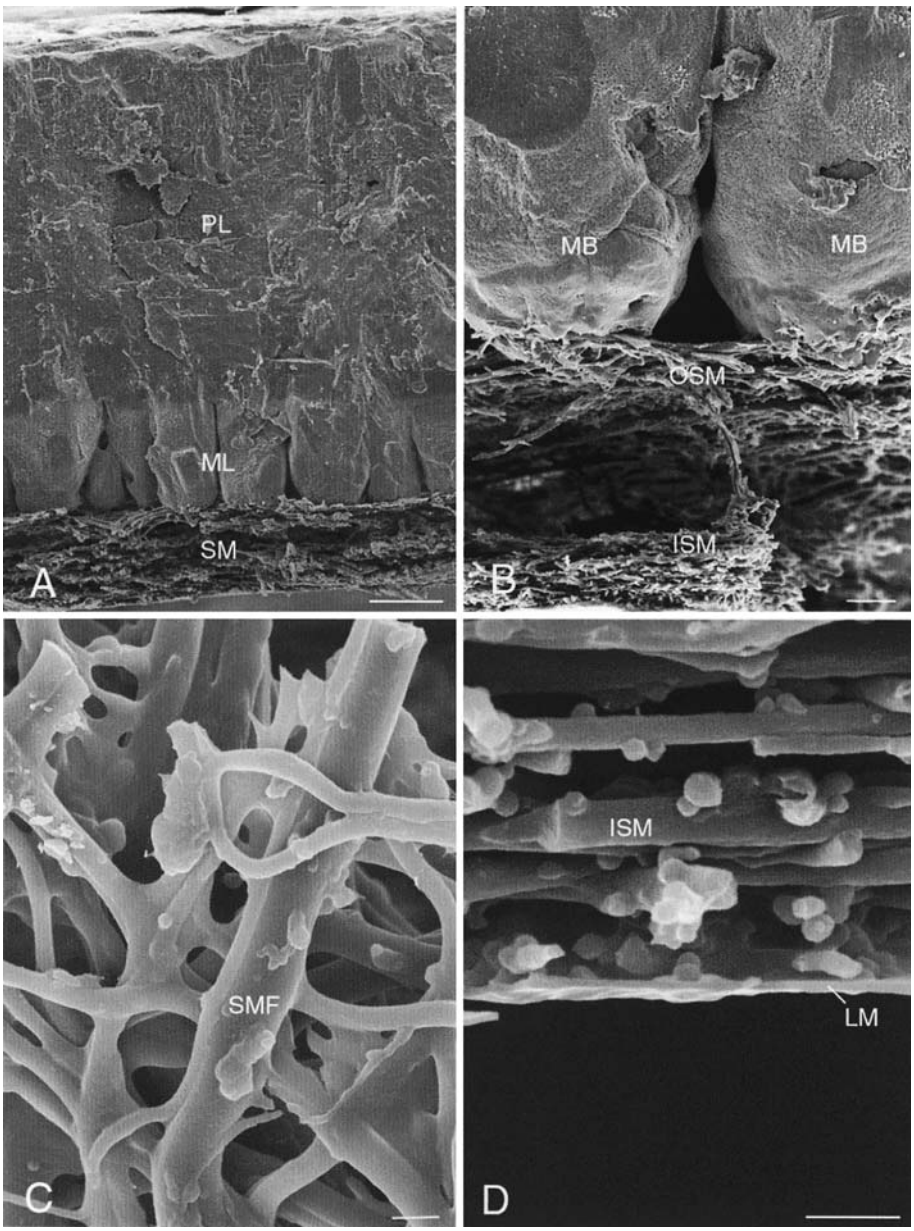

Fig. 1 Scanning electron micrographs illustrating the morphology of the eggshell and eggshell membranes. **a** Eggshell cross-fractured to reveal the shell membrane (*SM*), mammillary layer (*ML*) and palisade layer (*PL*). **b** Higher magnification of the membrane–mammillary body interface. Outer shell membrane fibres (*OSM*) insert into the tips of the mammillary bodies (*MB*). Inner shell membranes (*ISM*). **c** Enlargement of the shell membrane fibres (*SMF*) to reveal their interwoven and coalescing nature. **d** Inner aspect of the inner shell membrane (*ISM*), demonstrating the limiting membrane (*LM*) that surrounds the egg white (here removed during sample preparation). *Scale bars* **a** 50 mm; **b** 20 mm; **c**,**d** 2 mm. Reprinted from [24], with permission from Elsevier

The incomplete egg then enters the uterus, or shell gland, where fluid is pumped into the albumen, causing it to swell to its final size. During the following 16- to 17-h period, eggshell mineralization occurs while the ovum slowly rotates within the shell gland (10–12 turns/h) [7]. Shell calcification occurs in the uterine fluid which bathes the forming egg; all ingredients of this process are secreted by the epithelial and mucosal cells that line the lumen of the uterus. The ionic and protein constituents of the uterine fluid change progressively during eggshell formation and can be subdivided into the stages of initiation (5 h), growth and rapid calcification (12 h) and termination (1.5 h) of eggshell mineralization [8].

The mineralized shell consists primarily of calcite, the most stable polymorph of calcium carbonate, and extends from the inner mammillary cone layer, through the central palisades and the outer vertical crystal layers [3, 8] (Fig. 1). The mammillary cone layer, $\sim$100 µm thick [2], is composed of a regular array of cones or knobs, into which are embedded the individual fibers of the outer eggshell membrane. Within the mammillary cone layer, microcrystals of calcite possess spherulitic texture and are readily dissolved to mobilize calcium to meet the needs of the growing embryo [3]. The palisades region, $\sim$300 µm thick, is made up of groups of columns (crystallites) composed of elongated calcite crystals tending to a preferential orientation

with either the (001) or (104) plane parallel to the shell surface [9, 10]. Variability is observed in crystal orientation which has been correlated with shell strength [10]. The outer region of the palisade layer is a tough structure made of large calcite crystals where the external impacts are absorbed by thin inter-crystalline organic layers that make intracrystalline crack propagation difficult [3]. This layer ends at the vertical single crystal layer which has a crystalline structure of higher density than that of the palisade region.

Shell pigments and eggshell cuticle are deposited on the surface of the immobilized egg during the last 1.5 h before oviposition (egg expulsion). The outermost shell layer is the eggshell cuticle, a non-calcified organic layer of variable thickness which may even be absent. A thickness range of 5–10 μm has been reported [11]. The cuticle is composed of glycoproteins, polysaccharides, lipids and inorganic phosphorus including hydroxyapatite crystals [12–14]. The cuticle is thought to play a role in controlling water exchange by repelling water or preventing its loss, and may function in limiting microbial colonization of the eggshell surface [1, 13]. This layer, as well as the outer portion of the calcified shell, contains eggshell pigments which serve as camouflage, temperature control and possibly in parental recognition [15]. Pores span the eggshell and permit the diffusion of metabolic gases and water vapor to allow proper embryonic development [16]. As expected, eggshell thickness varies between bird species. For example, chicken eggshells are approximately 0.3–0.4 mm thick while the ostrich shell is 2.5 mm thick [17].

Biochemistry of eggshell matrix proteins

The eggshell mineral is associated with an organic matrix composed of proteins, glycoproteins and proteoglycans, termed "eggshell matrix proteins", which are progressively incorporated from the precursor milieu (uterine fluid) during calcification. Their function is thought to influence the fabric of this biomaterial and/or to participate in its antimicrobial defenses. These non-mineral constituents represent about 2% by weight of the calcified eggshell, and can be released for study by demineralization of the eggshell by calcium chelation (EDTA or EGTA) or acid demineralization (acetic acid or HCl), yielding soluble and insoluble constituents. A complex array of distinct protein bands was demonstrated in the soluble intra- and extramineral compartments by 1D-electrophoresis (SDS–PAGE) [18, 19], and in the precursor uterine fluid, showing different patterns between the three stages of the eggshell calcification process (initial, growth and terminal) [20]. In contrast, the insoluble components which are not soluble in SDS remain relatively uncharacterized (but see [21, 22]). N-terminal sequencing of the electrophoretic bands allowed the egg white proteins ovalbumin, lysozyme and ovotransferrin to be identified [23–25]. N-terminal and internal amino acid sequencing of other protein bands revealed that they did not correspond to previously identified proteins and these have been subjected to more intensive investigation. Purification schemes using ion exchange (diethylaminoethyl (DEAE)–Sepharose and carboxymethyl (CM)–Sepharose) and hydroxyapatite were developed to isolate ovocleidin-17 [26] and ovocalyxin-32 [27] from eggshell extracts.

Other eggshell matrix proteins were characterized by a combination of molecular cloning, immunochemistry and bioinformatics. A cDNA library from pooled RNA extracted from chicken uteruses that were harvested during the mid-phase of shell calcification was successfully screened to clone novel eggshell matrix proteins [28, 29]. Expression screening of this library, using polyclonal antisera raised to partially purified eggshell matrix proteins, allowed clones with the corresponding cDNA sequences to be identified and sequenced. The conceptual amino acid sequence was compared to partial amino acid sequencing data for proteins present in uterine fluid and eggshell extracts. This method allowed the identification of two novel eggshell matrix proteins, ovocleidin-116 [28] and ovocalyxin-36 [29]. Another associated approach was to compare the available expression sequence tag (EST) sequences to partial protein or nucleotide sequences from egg components. This method was successfully used to identify a 32-kDa band abundant in uterine fluid at the terminal phase of shell calcification (ovocalyxin-32) [30].

Such studies led to the concept that eggshell matrix protein components form three characteristic groups:

(1) "Egg white" proteins which are also present in the eggshell—these include ovalbumin, the most abundant egg white protein [23], lysozyme, an antimicrobial protein with hydrolytic activity against peptidoglycans on cell walls of Gram-positive bacteria [24] and ovotransferrin, which sequesters iron necessary for bacterial growth [25];

(2) Ubiquitous proteins that are found in many tissues—examples are osteopontin, a phosphorylated glycoprotein present in bone and other hard tissues [31–36], and clusterin, a widely distributed secretory glycoprotein that is also found in chicken egg white [37];

(3) Eggshell-specific matrix proteins unique to the shell calcification process that are secreted by cells in specific regions of the oviduct where eggshell mineralization is initiated (red isthmus) and continues to completion (uterus). It is these "eggshell-specific matrix proteins" which are the primary focus of this review. These matrix components are termed

ovocleidins (ovo, Latin: egg; kleidoun, Greek: to lock in, implying a functional role) or ovocalyxins (ovo, Latin: egg; calyx, Latin: shell, referring to their shell location), with distinction based on apparent molecular weight by SDS–PAGE when initially characterized.

Recently, a high-throughput tandem-mass spectrometry approach (MS/MS) identified more than 500 eggshell matrix proteins [38], including the most abundant proteins that were already known (above). It is highly unlikely that all these 520 proteins perform eggshell-specific functions or are involved in eggshell assembly. The majority of them are proposed to be remnants of previous stages of egg formation occurring in proximal segments of the oviduct, or intracellular proteins released by breakdown of the cells lining the oviduct during normal turnover [38]. According to this hypothesis, all proteins that are present in the uterine fluid during the calcification process become assimilated into the eggshell, many of them in a non-specific manner. However, the "eggshell-specific" proteins that are described in this review are abundant components of the eggshell matrix and are highly likely to be relevant to eggshell function. Supportive evidence for an eggshell-specific role would be: restricted high level expression in a limited oviduct segment, up-regulation of expression in synchrony with movement of the forming egg through the oviduct, demonstration of a secretory process (i.e., signal peptide, colloidal gold immunocytochemistry to demonstrate secretion granule localization) and secretion during eggshell formation, and finally, evidence for a role in eggshell function (i.e., calcification, antimicrobial protection). Many of these criteria have been met by the ovocleidins and ovocalyxins that are described in this review.

Two possible roles for eggshell-specific matrix proteins have been proposed; both reflect the protective function of the eggshell in avian reproduction: regulation of eggshell mineralization and antimicrobial defence. Egg calcification occurs in three distinct phases (initiation, active calcification, and termination of shell calcification), while the egg is bathed by the acellular uterine fluid containing the ionic and organic precursors of the eggshell. Each phase of shell mineralization is associated with a specific protein electrophoretic profile for the uterine fluid, suggesting that these molecules play specific roles during the calcification process [20].

Biomineralization

In general, the soluble matrix proteins of calcium carbonate biomaterials modify crystal growth, and therefore regulate the macroscopic properties of the resulting bioceramic. For example, in the mollusk shell, specific proteins control phase switching between the calcite and aragonite forms of calcium carbonate [39, 40]. A number of experimental observations support the role of the eggshell matrix proteins in determining the fabric of the eggshell and therefore influencing its resulting mechanical properties. Proteins in the uterine fluid modify the kinetics of calcium carbonate precipitation in vitro [41, 42]. The lag time for calcium carbonate precipitation is reduced by the uterine fluid from the initial and growth stages of eggshell mineralization, suggesting that these matrix precursors promote crystal nucleation. To a lesser extent, the uterine fluid collected during the growth phase also enhances precipitation kinetics. In contrast, the total uterine fluid harvested at the terminal stage of calcification inhibits calcite precipitation [19]. In agreement with these observations, partially purified eggshell matrix proteins inhibit calcium carbonate precipitation and alter patterns of calcite crystal growth, leading to morphological modifications of rhombohedric calcite crystals grown in vitro [19, 42]. Studies to investigate the effect of purified eggshell matrix proteins on calcium carbonate crystallization and crystal growth are described in the next section.

Antimicrobial protection

Protein extracted from the eggshell of the domestic chicken demonstrated antibacterial activity against *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enteritidis* [43]. Ovary and oviduct tissue extracts of the domestic hen were found to show antimicrobial activity against both Gram-positive and Gram-negative bacteria [44]. Moreover, eggshell cuticle protein extracts from a number of domestic and wild bird species demonstrated antimicrobial activity against *P. aeruginosa*, *B. subtilis* and *S. aureus* [45, 46]. Therefore, antimicrobial activity has been identified in protein extracts from avian eggshell and in particular outer eggshell; however, the identity of specific proteins responsible for the activity remains to be fully elucidated (see next section: "Eggshell-specific matrix proteins").

Eggshell-specific matrix proteins

Ovocleidin-17

Ovocleidin-17 (OC-17) was the first eggshell-specific matrix protein to be isolated and characterized following its chromatographic purification after eggshell decalcification [26]. Using an antibody raised to the purified protein, immunohistochemistry revealed that it is secreted by the tubular gland cells in the shell gland; within the shell, it is distributed throughout the shell matrix, but concentrated in the mammillary bodies [26]. The protein sequence contains

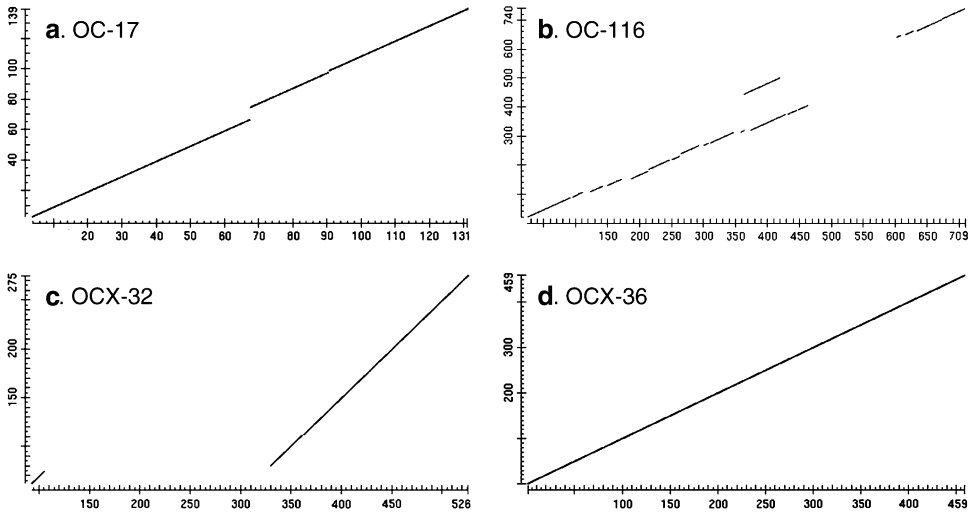

Fig. 2 Dot matrix plots to visualize regions of homology between orthologous chicken (*Gallus gallus*) and Zebra Finch (*Taeniopygia guttata*) eggshell matrix proteins. The NCBI align 2 sequences (bl2seq) tool was used. In each *panel*, the ordinate depicts the chicken sequence, while the Zebra Finch sequence is on the abscissa. The following accession sequences were compared (chicken, Zebra Finch): **a** *OC-17* (Q9PRS8, XP_002189493); **b** *OC-116* (NP_989900, XP_002190429); **c** *OCX-32* (NP_989865, XP_002186694); **d** *OCX-36* (Q53HW8, XP_002192664)

142 amino acids including 2 phosphorylated serines, Ser-61 and -67 [47]. Moreover, the protein also occurs in the eggshell soluble organic matrix as a minor form which is glycosylated at Asn-59 (23 kDa, OC-23) [48]. Glycosylation occurs at the N-glycosylation site consensus sequence, N-A-S which contains Ser-61. In the glycosylated form of the protein, Ser-61 is not phosphorylated, indicating that these modifications are mutually exclusive. In addition, peptides without modification at Asn-59 or Ser-61, with phosphorylation of Ser-67 only, or with no phosphorylation at all, have been detected [49]. The function of these modifications remains unknown but the phosphorylation sites are preserved in closely related proteins isolated from other avian eggshells (see below) suggesting their importance.

Detailed studies have identified homologous eggshell matrix proteins in shell from a diverse number of avian species. Comparison of their primary sequences revealed that ansocalcin (goose), struthiocalcin-1 and -2 (SCA-1 and -2; ostrich), dromaiocalcin-1 and -2 (DCA-1 and -2; emu) and rheacalcin-1 and-2 (RCA-1 and -2; rhea) form two groups based on sequence identity, serine phosphorylation and conservation of cysteine residues [17, 50, 51]. Goose ansocalcin aligns reasonably well with proteins of group 1 (63–70% identity with SCA-1, DCA-1, and RCA-1), but OC-17 has much less sequence identity with group 2 where it is placed (37–39% with SCA-2, DCA-2, and RCA-2). It remains unclear why ratites differ from goose and chicken in that they possess two forms of the *"-calcin"* matrix protein as their predominant eggshell matrix proteins. It is suggested that these differences are due to loss of one gene in modern birds (goose, chicken) [51], occurring after the divergence of paleognathae (ratites and tinamous) from neognatae (the ancestors of all other modern birds) more than 100 million years ago [52]. It can be proposed that homologous C-type lectin eggshell matrix proteins will be found in the shells of all other bird species (i.e., neoaves). One such candidate in the genome of the Zebra Finch (Accession: XP_002189493; predicted: similar to regenerating islet-derived family, member 4, *Taeniopygia guttata*) has been identified by automated gene prediction (Fig. 2a). In contrast, sequencing of the chicken genome (May 2006, version 2.1 release, 95% complete, 7.1× coverage) has not yet identified the OC-17 gene. Database searches with these eggshell protein sequences reveal that they belong to a heterogeneous group of proteins consisting of a single C-type lectin domain (CTL) and display sequence homology to members of this family such as mammalian Reg (regenerating islet-derived) proteins, pancreatic stone protein (lithostathine), fish Type II antifreeze proteins and anticoagulant proteins from snake venom [53]. The X-ray structure of OC-17 has been determined and reveals a mixed alpha helix/beta sheet structure and verifies the C-type lectin-like domain [54, 55]. Preliminary X-ray crystallographic studies of struthiocalcin-1 are underway [56].

The properties of purified OC-17 and its goose homolog (ansocalcin), and their influence upon calcite crystallization patterns have been investigated and compared [50, 55, 57, 58]. Functionally, OC-17 and ansocalcin do not appear to be completely equivalent in their effect on calcite crystal growth in vitro [55, 57]. Ansocalcin showed reversible concentration-dependent aggregation in solution, and was reported to induce pits on growing calcite rhombohedral faces at lower concentrations (<50 μg/ml) and to nucleate polycrystalline aggregates of calcite crystals at higher concentrations [50]. Aggregated ansocalcin may act as a template for the nucleation of calcite crystal aggregates [57]. However, under the same conditions, OC-17 was not observed to aggregate in solution nor induce the nucleation of calcite aggregates. Nevertheless, under different experimental conditions, Reyes-Grajeda et al. [55] reported that OC-17 could modify the crystalline habit of calcium

carbonate and the pattern of crystal growth at concentrations of 5–200 μg/ml. However, the observation that OC-17, but not ansocalcin, is largely destroyed by treating eggshell powder with bleach suggests a different intramineral location, or a different kind of interaction with mineral, for these proteins [58]. OC-17 and SCA-1, but not SCA-2, are reported to interact directly with carbonate anion, as a potential mechanism accounting for different effects upon calcite nucleation and crystal growth [59].

As previously mentioned, OC-17 shows significant similarity to the Reg family of C-type lectin proteins. Mammalian Reg III homologs (mouse Reg III gamma and human HIP/PAP) possess antimicrobial activity against Gram-positive bacteria [60]. OC-17 and ansocalcin were investigated to determine whether they possess antimicrobial activity [61]. A micro-broth dilution assay indicated that purified OC-17 and ansocalcin each inhibited the growth of Gram-positive bacteria, with OC-17 exhibiting greater activity. Both proteins were more active in the presence of Ca^{2+}. A pull-down assay demonstrated that OC-17 and ansocalcin interact with bacterial cell walls and cell wall peptidoglycans [61]. Comparison of the protein structures of OC-17 and ansocalcin (modeled on the X-ray structure determined for OC-17 [55]) revealed a broad distribution of positive charges in each molecule, similar to that found in cationic antimicrobial peptides [62] (Fig. 3).

OC-17 is an abundant eggshell-matrix specific protein (40 μg/g shell) [63]. Sensitive proteomic analyses of the chicken egg yolk plasma, egg white and vitelline membrane detected low levels of OC-17 [64–66].

Ovocleidin-116

Ovocleidin-116 (OC-116) was the first eggshell matrix protein to be cloned, by expression screening a uterine library using an antibody raised to the abundant 116-kDa protein observed in hen uterine fluid during the active calcification phase of shell formation [28]. OC-116 is a major eggshell matrix protein, estimated at 80 μg/g eggshell powder [63]. Western blotting for OC-116 revealed that only the uterine portion of the oviduct, where shell calcification occurs, was immunopositive and that 180- and 116-kDa immunoreactive bands were detected in uterine tissue, uterine fluid and eggshell matrix. The N-terminus of the mature protein and conceptual translation product from cDNA correspond to that previously reported for a 200-kDa eggshell matrix proteoglycan that is converted to 120 kDa by chondroitinase ABC treatment [67]. Therefore, OC-116 is the core protein (predicted 75 kDa) corresponding to the doublet bands of an eggshell dermatan sulfate proteoglycan (116–120 and 180–200 kDa). It is hypothesized that the 180- to 200-kDa form of OC-116 corresponds to the N-glycosylated core protein with

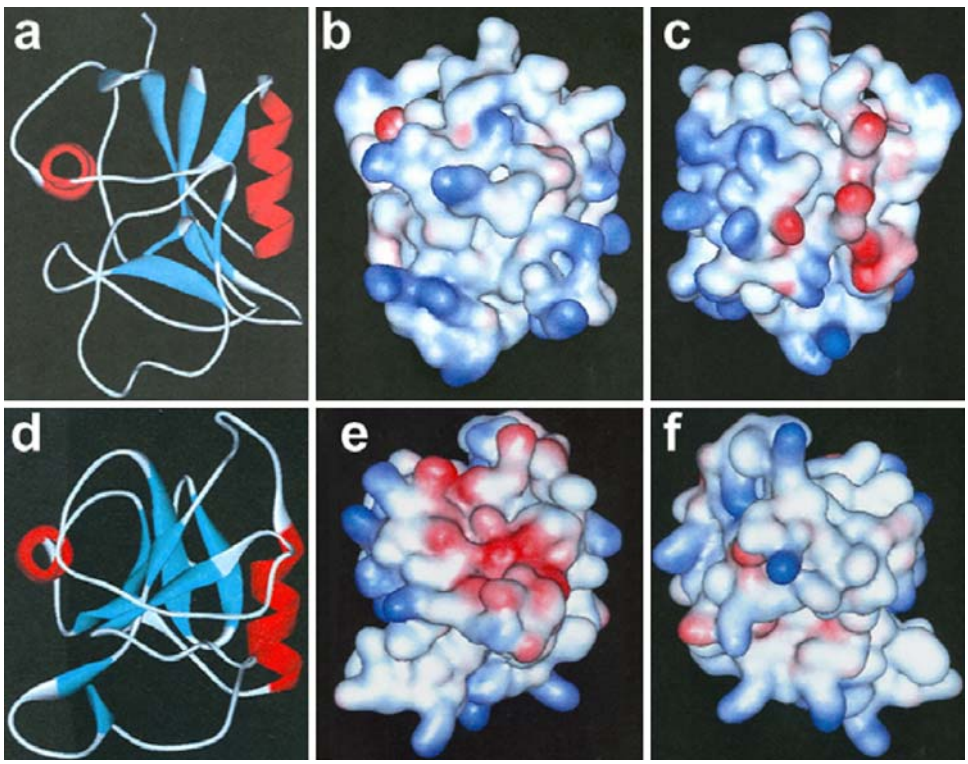

Fig. 3 Three-dimensional structures of avian eggshell CTL proteins. **a** Crystal structure of OC-17. **b** Surface structure of OC-17 in the same orientation as in (**a**). **c** Surface structure of OC-17 that is rotated 180° clockwise around the *Y*-axis of (**a**). **d** Molecular model of ansocalcin. **e** Surface structure of ansocalcin model in the same orientation as in **d**. **f** Surface structure of ansocalcin that is rotated 180° clockwise around the *Y*-axis of (**d**). In **a** and **d**, protein secondary structures are indicated in *blue* (b-strand) and *red* (a-helix). The *blue* and *red* colors in the surface plot indicate the distribution of positive and negative charges, respectively. Reprinted from [61], with permission from Elsevier

attached glycoaminoglycans, while the 116- to 120-kDa form corresponds to the protein without glycoaminoglycans [28]. Sequencing of peptides purified from protease-treated eggshell extract reveal that both predicted N-glycosylation sites are modified; however, while Asn-62 is entirely glycosylated, Asn-293 is only marginally occupied [63]. Detailed analysis of the carbohydrate structures attached to Asn-62 revealed 17 different oligosaccharide structures [68]. High-mannose, core-fucosylated and peripherally fucosylated structures were present. The relatively rare lacdiNAc (GalNAcbeta1–4GlcNAc) motif was detected in more than half the structures, while the lacNAc (Galbeta1–4GlcNAc) motif, which is the more frequent motif in mammals, only occurred in 3 of the 17 glycoforms. Glycoaminoglycans associated with OC-116 have not yet been characterized.

OC-116 is phosphorylated to a variable and partial extent on at least 22 serine and threonine residues. Two sites that were frequently identified with different cleavage methods were Ser-444 and Thr-664 [49]. Ultrastructural immunocytochemistry indicates that OC-116 is synthesized and secreted from the granular cells of the uterine epithelium, and is incorporated into, and widely distributed throughout, the palisade region of the calcified eggshell [28]. Such localization studies do not distinguish between the differentially phosphorylated, N-glycosylated or glycanated forms of OC-116, nor would possible differences in eggshell distribution between the 116 and 180 kDa forms be detected by this technique. Crystal growth studies have shown that pure glycoaminoglycans affect calcite morphology, leading to crystal elongation [69], suggesting that the sulfated form of OC-116 (MW 220 kDa) would influence eggshell mineralization via electrostatic interactions.

The sequence of OC-116 is quite unlike those of other calcified tissue proteoglycans [28, 63]. Low stringency BLAST searching with the OC-116 protein sequence generated restricted and poorly significant alignments to mammalian and chicken collagens (types I, II, VII and IX), human perlecan (heparan sulfate proteoglycan), chicken aggrecan (chondroitin sulfate proteoglycan), chicken bone sialoprotein and lustrin A (component of mollusk shell extracellular matrix). No homology with avian versican was apparent by BLAST searching, although both forms of OC-116 are recognized by a monoclonal antibody that is specific for an epitope on the core protein of avian versican [67]. OC-116 does not possess common structural characteristics that are found in aggrecan, PG-M/versican, neurocan and brevican [i.e., domains that are EGF-like, C-type lectin-like and complement regulatory protein (CRP)-like] [70]. Proteoglycans have the potential to function in biomineralization since their glycosaminoglycan units consist of repeating disaccharides with carboxylate and/or sulfate moieties. It remains to be determined whether the underlying mechanisms by which OC-116 acts are similar to those by which proteoglycans promote cartilage calcification and collagen mineralization in vitro [71, 72].

The release of the chicken genome sequence in 2004 revealed that the chromosomal localization of OC-116 is adjacent to that of osteopontin on chromosome 4 [73]. These 2 genes are contiguous with other mineralization-specific genes (bone sialoprotein, dentin matrix protein 1) that form the SIBLING (small integrin-binding ligand, N-linked glycoprotein) mineralization gene locus first reported in mammalian genomes (Fig. 4). This correspondence reflects synteny between avian and mammalian genomes. Moreover, investigations into the evolutionary genetics of vertebrate tissue mineralization suggest that OC-116 and other SIBLING proteins are members of the secretory calcium-binding phosphoprotein (SCPP) family that function in tetrapod mineralization [74, 75]. Based on its position within this gene locus, OC-116 is predicted to

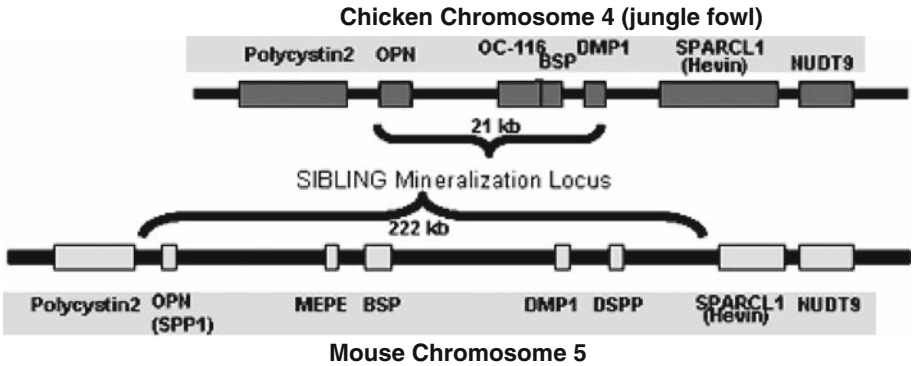

Fig. 4 Schematic depiction of the SIBLING mineralization gene loci in chicken and mouse. The Zebra Finch SIBLING locus displays an identical organization and relative placement of its OC-116 (Accession XP_002190429) as in chicken. The OC-116 gene occupies the corresponding position of the mammalian MEPE gene, suggesting that OC-116 and MEPE are orthologs. *Sibling* small integrin-binding ligand, N-linked glycoprotein, *OPN* (*SSP1*) osteopontin, *MEPE* matrix extracellular phosphoglycoprotein, *BSP* bone sialoprotein, *DMP1* dentin matrix protein 1, *DSPP* dentin sialophosphoprotein

be the avian ortholog of mammalian MEPE (matrix extracellular phosphoglycoprotein) (Fig. 4). In support of this suggestion, BLAST 2-sequence testing reveals that the N-terminus of chicken OC-116 possesses about 30% identity with the *Homo sapiens* MEPE protein sequence. As expected, the SIBLING gene locus (OPN, OC-116, BSP and DMP-1) also exhibits synteny between the two avian genomes that have been sequenced to date: *Gallus gallus* and *Taeniopygia guttata* [76]. There is at best about 40% identity between the amino acid sequences of chicken and Zebra Finch OC-116; the homology is strongest between the N-terminal halves and at the C-termini (Fig. 2b). Thus, while OC-116 has evolved to fulfill a specific function in mineralization of the calcitic eggshell, there are large differences between avian forms of the protein. Further study of common features of different avian OC-116s should help to define its features that are important for mineralization.

Proteins originating from the SCPP genes have a common characteristic; they bind calcium ions via acidic amino acids such as Glu, Asp and phospho-Ser [74]. One member, osteopontin, is also an eggshell matrix protein [31–36], and regulates calcification in vertebrate biominerals such as bone and teeth [77]. In the eggshell, osteopontin may function during mineralization by inhibiting calcium carbonate precipitation in a phosphorylation-dependent manner [32, 78]. However, OC-116 is uniquely specialized as an avian member of this mineralization-specific family, supporting the hypothesis that it has a key role in mineralization of the avian shell. A strong genetic linkage was detected between single nucleotide polymorphisms (SNPs) in the OC-116 gene during studies to investigate possible correlation between eggshell biomechanical properties and eggshell matrix protein SNPs. SNPs in the OC-116 gene are significantly associated with the eggshell elastic modulus and thickness, and with egg shape [79].

Recent studies report new possible locations for OC-116 protein. Proteomic analysis of the chicken egg yolk plasma determined its presence in the yolk [64]. OC-116 was recently detected in young chick cortical bone, laying hen medullary bone and growth plate hypertrophic chondrocytes suggesting a possible role in calcium phosphate mineralization [80].

Ovocalyxin-32

Ovocalyxin-32 (OCX-32) was originally identified as a 32-kDa uterine fluid protein that is abundant in the terminal phase of shell formation [25, 27]. Sequencing of peptides derived from the purified protein allowed expressed sequence tag sequences (ESTs) to be identified that were assembled to yield a full-length composite sequence whose conceptual translation product contained the complete amino acid sequence of OCX-32. OCX-32 is expressed at high levels in the uterine and isthmus regions of the oviduct and is secreted by the surface epithelial cells that line the lumen [25]. In the eggshell, OCX-32 localizes to the outer palisade layer, the vertical crystal layer, and the cuticle of the eggshell, in agreement with its demonstration by Western blotting at high levels in the uterine fluid during the termination phase of eggshell formation [21, 25, 27]. A study of eggshell phosphoproteins identified phosphorylation of OCX-32 at serines and threonines between position 257 and 268, but exact sites were not determined [49].

OCX-32 protein possesses limited identity (32%) to two distinct mammalian proteins, latexin, and retinoic acid receptor responder (tazarotene induced) 1 (RARRES1), that are modestly related to each other (about 30% sequence identity). Latexin is an inhibitor of carboxypeptidase A activity, and is expressed in rat cerebral cortex and mast cells [81, 82]. It plays a role in inflammation, as it is expressed at high levels and is inducible in macrophages in concert with other protease inhibitors and potential protease targets. Its structural domains share a cystatin fold architecture found in proteins that inhibit cysteine proteases [83]. RARRES1 was originally isolated from human skin. Its expression is upregulated by tazarotene as well as by retinoic acid receptors; it is downregulated in human neoplastic cells as a result of methylation of its promoter and CpG island [84]. RARRES1 has been implicated in the therapeutic effects of retinoic acid in psoriasis and in tumor suppression [85, 86]. There is conservation of corresponding exon boundaries between the aligned protein sequences of OCX-32, latexin and RARRES1, suggesting that there is an evolutionary connection between these mammalian proteins and OCX-32 (i.e., gene duplication followed by divergence) [87]. The human latexin/RARRES1 genes are located within 60 kb of each other on chromosome 3.

The timing of OCX-32 secretion into the uterine fluid has been interpreted to suggest that it plays a role in the termination of eggshell calcification [20]. This hypothesis originated from the observations of morphological changes in calcite crystals by uterine fluid collected during the terminal phase of calcification and the location of OCX-32 in the mineral pellet after its precipitation with calcium carbonate in vitro from fresh uterine fluid [41, 42]. Studies with purified OCX-32 are necessary to investigate this possibility. A study in commercial pedigree hens for eggshell matrix candidate gene associations with eggshell quality measurements found that OCX-32 SNPs are significantly associated with mammillary layer thickness [79]; the basis for this association is unclear since OCX-32 is predominantly localized to the outer eggshell. An interesting recent observation is that the OCX-32 gene is expressed at higher levels in a low egg production strain

(compared to a high production strain) of Taiwanese country chickens [88].

Another suggestion for OCX-32 function is based on OCX-32 homology to the carboxypeptidase A inhibitor latexin, and demonstration that proteinase inhibitors such as SLPI and elafin possess antimicrobial activity through inhibition of microbial proteases [89]. OCX-32 has a predominant localization in the cuticle, the layer in direct contact with the environment and exterior pathogens, and is ideally positioned as a "first-responder" molecule against bacterial colonization of the eggshell. To obtain sufficient material for further studies of its function, recombinant OCX-32 protein was expressed in *E. coli* [90]. The protein was extracted from inclusion bodies and purified by sequential DEAE Sepharose and Ni^{2+} metal ion affinity chromatographies as a 58 kDa GST-fusion protein. The refolded GST–OCX-32 significantly inhibited bovine carboxypeptidase activity and also inhibited the growth of *B. subtilis*. These results support the notion that OCX-32 provides antimicrobial protection for the egg.

Proteomic studies have recently noted low levels of OCX-32 in egg white [66] and in the vitelline membrane [65]. Using polyclonal antiserum raised to chicken OCX-32, a 32-kDa immunoreactive band was detected by Western blotting in eggshell extracts from a variety of domestic and wild anseriform and galliform species, suggesting conservation of primary sequence across avian orders [45, 46]. This prediction is supported by a fair degree of similarity between the chicken and Zebra Finch homologous proteins (OCX-32, RARRES1, 56% identity between the respective C-terminal regions) (Fig. 2c).

Ovocalyxin-36

Ovocalyxin-36 (OCX-36) is a prominent 36-kDa protein present in the uterine fluid collected during the active calcification stage of shell mineralization. Antibodies raised to the uterine protein were used to expression-screen a hen uterine library, and a novel clone was identified which was the basis for additional rounds of hybridization-screening. The resulting consensus sequence was subsequently assembled with public database EST's to obtain complete full-length cDNA [29]. The protein is only detected in the regions of the oviduct where eggshell formation takes place (isthmus and uterus). Moreover, the uterine OCX-36 message, quantified by real time RT-PCR, is strongly upregulated during eggshell calcification [29]. OCX-36 localizes to the calcified eggshell predominantly in the inner part of the shell, and largely to the shell membranes. OCX-36 protein sequence is 20–25% identical to mammalian proteins associated with the innate immune response, such as lipopolysaccharide-binding proteins (LBP), bactericidal permeability-increasing proteins (BPI) and palate, lung and nasal epithelium clone (Plunc) family proteins [27]. The genomic organization of LBP, BPI and OCX-36 appear to be highly conserved, suggesting an evolutionary link [27]. These observations suggest that OCX-36 is a novel and specific chicken eggshell protein related to the superfamily of LBP/BPI and Plunc proteins. These proteins are well known in mammals for their involvement in defense against bacteria. They belong to the superfamily of proteins known to be key components of the innate immune system which act as the first line of host defense [91]. LBP proteins initiate the inflammatory host response upon the detection of a pathogen [92]. LBP binds the lipid A component of the lipopolysaccharide (LPS) layer of Gram-negative bacteria and transfers them to CD14, an LPS receptor [93]. BPI also binds LPS, followed by permeabilization of the cytoplasmic membrane and a decrease in the electrochemical gradient of the bacterial cell leading to death [94]. OCX-36 may therefore participate in natural defense mechanisms that keep the egg and oviduct free of pathogens.

OCX-36 is also detected in the vitelline membrane [65]. Chicken and Zebra Finch OCX-36 exhibit a large degree of similarity throughout the protein sequence (56% identity), as seen in Fig. 2d, indicating greater conservation between these species for this matrix protein compared to OC-17, OC-116 or OCX-32.

Other eggshell-specific proteins

Recently, it was reported that 21- and 25-kDa components of the eggshell matrix/uterine fluid have been cloned [95]. Analysis of the 21-kDa protein sequence showed significant homologies with proteins containing the BRICHOS domain to which a chaperone-like function has been ascribed [96]. The 25-kDa protein contains two protease inhibitor domains. One is a WAP type that is also present in lustrin A, a matrix protein from the nacreous layer of the shell and mother of pearl of mollusks [97].

Non-avian eggshell matrix proteins

In eggs of turtles, the eggshell consists of two parts: a fibrous shell membrane adjacent to the albumen and a calcareous layer composed of the aragonite polymorphic form of calcium carbonate. Pelovaterin is an anionic peptide present in the aragonitic eggshells of the soft-shelled turtle (*Pelodiscus sinensi*), an animal so-called because its carapace is leathery and pliable [98]. Pelovaterin was purified from the soluble organic matrix by RP-HPLC. The amino acid sequence, 42 residues in length, shows no homology to any known protein. This sequence is rich in Gly, Cys, Ser and Val with N-terminal

and C-terminal sequences containing hydrophilic residues. Dynamic light scattering, fluorescence emission spectroscopy and circular dichroism indicated that pelovaterin is monomeric in lower concentrations and aggregates at high concentrations [98].

Pelovaterin seems to have a role in the biomineralization of the soft-shelled turtle eggshell. In vitro calcium carbonate crystallization tests show that pelovaterin induces the formation of the vaterite polymorph of calcium carbonate, alters crystal morphology and increases the mineral growth rate [98]. Crystal nucleation may be related to the aggregation of pelovaterin at high concentration. More recently, the same researchers have demonstrated that pelovaterin stabilizes the metastable vaterite phase of the eggshell, but under certain recrystallization conditions can induce and stabilize aragonite as the dominant polymorph [99].

NMR structural analysis indicated that pelovaterin contains a hydrophobic core and a structure similar to cationic antimicrobial peptides such as defensins [99]. Antimicrobial assays revealed that pelovaterin was very effective against the Gram-negative bacteria *P. aeruginosa* and *Proteus vulgaris,* possessed moderate activity against *Proteus mirabilis* and *S. aureus*, but showed very little activity against *E. coli* and *Enterobacter aerogenes* [99]. Scanning electron microscopy to investigate its bactericidal activity against *P. aeruginosa* and *Proteus vulgaris* revealed that treatment with 10 μM pelovaterin produced pronounced wrinkling, surface roughening, and blebbing of the bacterial membrane and the majority of the cells lost their membrane integrity.

The results demonstrate the adaptability of an eggshell matrix protein to perform multiple tasks: calcium carbonate polymorph discrimination and protection of the contents of the egg against bacterial invasion.

Conclusion/perspectives

The majority of constituents of the chicken eggshell have been identified. Future effort to compare and contrast the chicken eggshell matrix proteins with those of other avian and non-avian eggshells will pay dividends to fully determine the function of the eggshell matrix proteins. Two functional roles have been proposed: (1) regulation of eggshell mineralization, and (2) antimicrobial protection of the egg and its contents. Bioinformatic studies with nucleotide and amino acid sequences contribute to the identification of putative function of proteins; however, sensitive proteomic methods increasingly reveal the minor presence of "eggshell-specific" proteins in other egg compartments although the functional impact at these sites remains to be determined. New information from studies with purified native or recombinant proteins are necessary for in vitro tests to gain insight into the role of each isolated matrix component, and eventually to learn how they may function synergistically. One important goal will be to determine the impact and importance of posttranslational modification of matrix components (glycosylation, glycanation, phosphorylation, etc.), which could greatly alter their properties and interactions. An emerging concept is the dual role (mineralization/antimicrobial protection) that certain eggshell matrix proteins can play. These investigations will continue to provide new insights into function of integrated defense strategies that operate at biomineralized barriers.

Acknowledgments The authors work has been supported by funding from Natural Sciences and Engineering Research Council (NSERC), Ontario Ministry of Agriculture, Food and Rural affairs (OMAFRA) and Poultry Industry Council (PIC) to M.T.H.

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