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Novel identification of matrix proteins involved in calcitic biomineralization

*Megan Rose-Martel, Sandy Smiley, Maxwell T. Hincke**

Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario K1H 8M5, Canada

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ABSTRACT

Calcitic biomineralization is essential for otoconia formation in vertebrates. This process is characterized by protein–crystal interactions that modulate crystal growth on an extracellular matrix. An excellent model for the study of calcitic biomineralization is the avian eggshell, the fastest known biomineralization process. The objective of this study is to identify and characterize matrix proteins associated with the eggshell mammillary cones, which are hypothesized to regulate the earliest stage of eggshell calcification. Mammillary cones were isolated from 2 models, fertilized and unfertilized, and the released proteins were identified by RP-nanoLC and ES-MS/MS proteomics. Proteomics analysis identified 49 proteins associated with the eggshell membrane fibers and, importantly, 18 mammillary cone-specific proteins with an additional 18 proteins identified as enriched in the mammillary cones. Among the most promising candidates for modulating protein–crystal interactions were extracellular matrix proteins, including ABI family member 3 (NESH) binding protein (ABI3BP), tiarin-like, hyaluronan and proteoglycan link protein 3 (HAPLN3), collagen alpha-1(X), collagen alpha-1(II) and fibronectin, in addition to the calcium binding proteins calumenin, EGF-like repeats and discoidin 1-like domains 3 (EDIL3), nucleobindin-2 and SPARC. In conclusion, we identified several cone-resident proteins that are candidates to regulate initiation of eggshell calcification. Further study of these proteins will determine their roles in modulating calcitic biomineralization and lead to insight into the process of otoconia formation/regeneration.

Biological significance

Biomineralization is essential for the development of hard tissues in vertebrates, which includes both calcium phosphate and calcium carbonate structures. Calcitic mineralization by calcium carbonate is an important process in the formation of otoconia, which are gravity receptor organs located in the inner ear and are responsible for balance and for sensing linear acceleration. Deficiencies in the regulation of their biomineralization can lead to otoconia degeneration and eventually benign paroxysmal position vertigo (BPPV), which is the main cause of vertigo in humans. Eggshell formation in chicken is one of the fastest known biomineralization processes and is an excellent model for the study of calcitic biomineralization. Cross-analysis of proteomic data from two mineralized models, fertilized and unfertilized chicken eggshells, identified proteins associated with the mammillary cones that are the sites of initiation of eggshell formation. We hypothesize

* *Corresponding author*. Tel.: +1 613 562 5800x8193; fax: +1 613 562 5434.
E-mail address: mhincke@uottawa.ca (M.T. Hincke).

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that these proteins regulate the earliest stages of eggshell calcification. The human homologs of these proteins are therefore potential candidates to play a role in otoconia biomineralization.

1. Introduction

Biomineralization is the process of directed mineral formation from supersaturated ion solutions, and is critical for the formation of biological hard tissues such as bone, teeth and calcified shells [1]. Calcitic biomineralization is essential for the formation of otoconia in vertebrates; these are calcium carbonate crystals located in the gravity receptor organs in the inner ear and are responsible for sensing linear acceleration and maintaining balance [2]. The formation of each otoconium is modulated by precise protein–crystal interactions between calcium carbonate and the otoconial membrane, which consists of Otolin-1, a member of the collagen X family, and five non-collagenous glycoproteins as well as keratin sulfate proteoglycan (KSPG) [2,3]. The most common cause of vertigo in humans is benign paroxysmal position vertigo (BPPV), a disease caused by the detachment of otoconia from the otolith organs [4]. This is especially common in elderly people who are prone to otoconia degeneration [5], which features mass reduction and structural damage [4]. Further studies are necessary to characterize the protein–crystal interactions that form and maintain the otoconia in order to develop strategies to slow or reverse degeneration.

Similar to otoconia formation, calcitic biomineralization leading to eggshell formation in birds occurs on the eggshell membranes, a highly cross-linked extracellular matrix containing cysteine-rich eggshell membrane proteins (CREMPs) and collagen types I, V and X [1,6,7] (Fig. 1B). Eggshell formation is one of the fastest known biomineralization processes; in chickens, complete formation represents deposition of approximately 6 g of calcium carbonate over a 17-hour period within the uterus [1]. The chicken calcified eggshell is an accessible model for the study of calcitic biomineralization.

Egg formation occurs with highly regulated temporal and spatial control; the beginning of eggshell deposition occurs with eggshell membrane fabrication while the forming egg passes through the white isthmus segment of the oviduct. Subsequently, the incomplete egg enters the distal red isthmus region where organic aggregates are deposited in a semi-periodic array on the surface of the outer eggshell membranes. Calcium carbonate deposition begins at these nucleation sites once the forming egg moves into the uterine portion of the oviduct, where it remains for approximately 17 h while the calcified mammillary cone (~100 μm) and palisade (~300 μm) layers are deposited; finally, the proteinaceous cuticle (~5–10 μm) is laid down [8–10] (Fig. 1A, B). The size and spacing of the mammillary cones influence the overall strength and biomechanical properties of the completed eggshell [11].

The mammillary cones are particularly relevant to embryonic development in the fertilized egg; the calcium reserve body located at the base of each cone contains reactive microcrystals of calcite which are more susceptible to dissolution and the

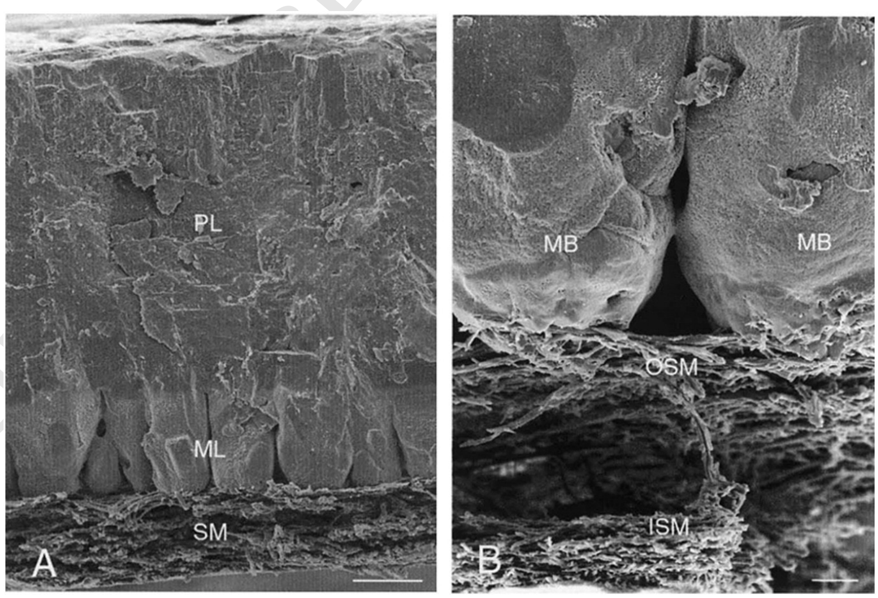

Fig. 1 – Scanning electron micrographs illustrating the morphology of the eggshell and eggshell membranes. (A) Eggshell cross-fractured to reveal the shell membrane (SM), mammillary layer (ML) and palisade layer (PL). (B) Higher magnification of the membrane/mammillary body interface. Outer shell membrane fibers (OSM) insert into the tips of the mammillary bodies (MB). Inner shell membranes (ISM). Scale bars: A, 50 μm and B, 20 μm.

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