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Short Communication

Observations on nascent matrix structures in embryonic cornea: Important in cell interactions, or merely vestiges of the lens surface?

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Abstract

Here we present some new observations on early stages in chick corneal development obtained by re-mining of datasets obtained via serial block face scanning electron microscopy. We focus on matrix cords, proteoglycan-rich structures of apparent ectodermal origin, emerging from the epithelial basal lamina, which extend proximally into the growing collagenous matrix destined to become the corneal stroma. Cords have no known function. In their earliest manifestation, we describe how they appear to run continuously from epithelium to the lens, in contact with both tissues and may therefore be simply vestigial structures, remaining from the earlier detachment of the lens from its parent ectoderm.

However, neural crest cells migrating to form the corneal endothelial monolayer appear to form close associations with cords via elaborate pseudopodial extensions. Presumptive endothelium and keratocytes, in the subsequent wave of neural crest cell influx, may conceivably utilise cords, as well as utilising collagenous fibrils of the interstitial matrix, as substrate cues in cell guidance, attachment and migration. The possibility also exists that cords fulfil a functional role in corneal morphogenesis via mechanotransduction through cell matrix interactions.

Keywords: Cornea; Stroma; Development; Matrix cords; Basal lamina; Lens

Abbreviations: 3D: Three Dimensions; SBF SEM: Serial Block Face Scanning Electron Microscopy; E: Embryonic day

Introduction

Historically the avian cornea has been a valuable model for studies of the development of a highly-organised lamellar connective tissue with specialised function. Its accessibility in the avian embryo and lesser availability in mammalian systems has meant that the majority of our knowledge of corneal development stems from observations on the chicken embryo [1-4]. Only quite recently, however, have advances in imaging techniques made it possible to observe the structure, organisation and emergence of this remarkable, regular matrix in three dimensions (3D) [5-8]. Some 3D studies on mammalian corneal development have also been reported [9,10]. In particular, the development of serial block face scanning electron microscopy (SBF SEM) [11,12], which allows high resolution imaging at the level of single collagen fibrils in the x-y plane and 3D reconstruction of large tissue volumes, has provided new insights into the involvement of both cell-directed and cell-independent mechanisms in the deposition of the collagenous corneal stroma [7,8]. In our previous study using these techniques, we considered intrinsic mechanisms which might generate the orthogonal arrangement of the very first fibrils of collagen, deposited by the ectoderm as micro-lamellae in the primary stroma [8]. We also focused on 'matrix cords', string-like proximal extensions of the epithelial basement membrane and presented a model, based on engineering principles, illustrating how they might contribute to the curvature of the corneal stroma. Matrix cords are enigmatic structures, with as yet no confirmed function in corneal development in spite of their discovery in the early pioneering studies of Hay and Revel [1]. A major advantage of the large datasets generated by SBF SEM is that they can be re-mined repeatedly for additional information to test new or alternative hypotheses. In this *Short Communication*, we present some further considerations of

our earlier data, focussing on matrix cords in the very earliest stages of corneal development in the chicken embryo. These observations, although not supported by large sample numbers, suggest a potential origin for these structures and support their putative role in matrix interactions with inwardly migrating neural crest cells destined to form the endothelium, in addition to interactions with presumptive keratocytes that we identified previously [7].

Materials and Methods

Tissues examined in this report were the same as those employed previously and from which data were presented in a recent publication [8]. In brief, corneas were isolated from the eyes of white Leghorn chicken embryos between days 4 and 6 of incubation (E4-E6), providing a total of 99 specimens. Following preservation in a paraformaldehyde/glutaraldehyde fixative, they were immersed sequentially in a series of heavy metal solutions, including osmium, uranium and lead, designed to impart high backscatter electron yield for imaging upon exposure to the electron beam of a scanning electron microscope. After thorough washing to remove surplus reagent, the tissue samples were dehydrated, embedded and polymerised in epoxy resin. Once orientated, trimmed and mounted on specimen pins, 16 specimens were imaged by SBF SEM (E4, n=8; E5, n=3; E6, n=5). Datasets, each comprising up to 1000 serial-images, were captured at 100 nm intervals, via sequential removal of a block-surface slice using an automated microtome (Gatan 3View) mounted inside the chamber of a Zeiss Sigma FEG scanning electron microscope. Image analysis and generation of 3D reconstructions were carried out using ImageJ/Fiji or Amira 6.2 software. Additional specimens were processed for examination in ultrathin section by transmission electron microscopy to visualise proteoglycans after glutaraldehyde fixation in the presence of the cationic dye cuprolinic blue, prior to dehydration and resin embedding.

Results

Observations by SBF SEM and cryo-section immunohistochemistry (here, and in unpublished studies by Koudouna and Ralphs), have confirmed the presence of the matrix structures we termed 'cords' throughout corneal development, from E4 to E18 in the avian cornea. They appeared perhaps most conspicuous at E6 (Figure 1), just following swelling of the primary stroma, when matrix density is yet to increase substantially. They extended throughout the entire depth of the growing stroma as tortuous strands normally positioned perpendicularly proximal to the epithelial basement membrane, but occasionally angled sharply and in contact with underlying migrating mesenchymal cells. Cords appeared by transmission electron microscopy to be rich in associated proteoglycans as evidenced by abundant filamentous structures, revealed as complexes with the cationic dye, cuprolinic blue (Figure 2). Proteoglycan filaments ran transversely and in parallel in relation to the major orientation of the cord, which itself appeared to be composed of multiple aligned microfibrils. At their points of origin within the epithelial basal lamina, the cord/proteoglycan filament arrangement was dissimilar to that in the basement membrane itself, which appeared more complex, presumably reflecting different polyanionic constituents.

At embryonic stages illustrating the earliest deposition of the primary stromal collagen fibrils, when the surface of the formative lens was but a few microns separated from its parent epithelium, matrix cords were already identifiable (Figure 3; Supplementary Video 1).

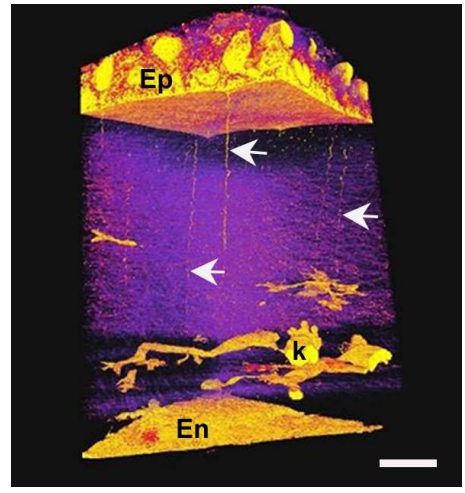

Figure 1: 3D reconstruction, made using Amira software, from 1000 serial images obtained by serial block face scanning electron microscopy of the developing cornea from the eye of a chick at embryonic day 6. Matrix 'cord' structures (arrows) extend from the basal lamina of surface epithelium (Ep) into the vicinity of presumptive keratocytes (k) invading the primary stroma, with endothelium (En) at its proximal limit. Bar = 20 µm.

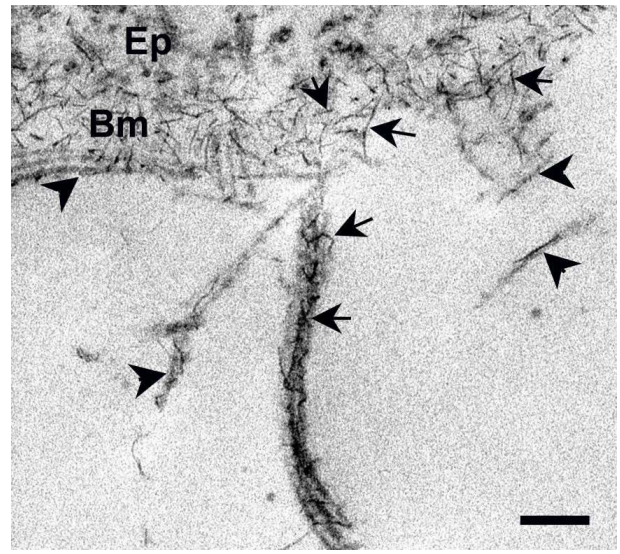

Figure 2: Transmission electron microscopy of chick cornea at E6 after contrast enhanced fixation with the cationic dye, Cuprolinic blue. A matrix cord descends from the epithelial cell (Ep) basement membrane (Bm). A complex array of dye-positive filaments (arrows), likely proteoglycans, are evident associated with the cord, basement membrane and nearby collagen fibrils (arrowheads). Bar = 250 nm.

These short strands of matrix occasionally appeared to extend continuously between, and in contact with, both the basal epithelium and the surface of the lens (Figure 3A-D), or alternatively to taper into the matrix at a point just distal to the lens (Figure 3E-H). At this stage also, the distal surface of the lens in some locations was associated with considerable amounts of basal lamina-like material (Figure 4).

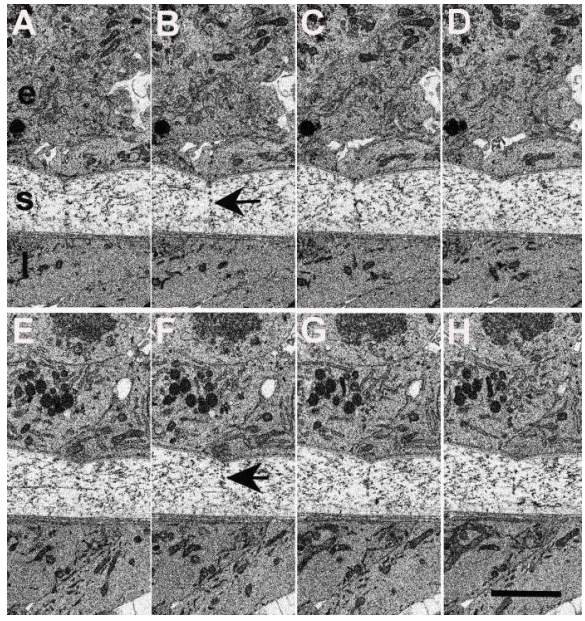

Figure 3: Two sequences of four serial images at 100 nm intervals from early E4 chick SBF SEM dataset. Corneal epithelium (e) and lens surface (l) are in close proximity, separated by early deposition of primary stroma (s). A single matrix cord, emerging from a protrusion in the epithelial basal lamina, is present in each sequence (arrows): in A – D, appearing continuous between epithelium and lens; in E – F, trailing above the lens surface. Bar = 10 µm. Both datasets are viewable in Video 1 Supplementary Information.

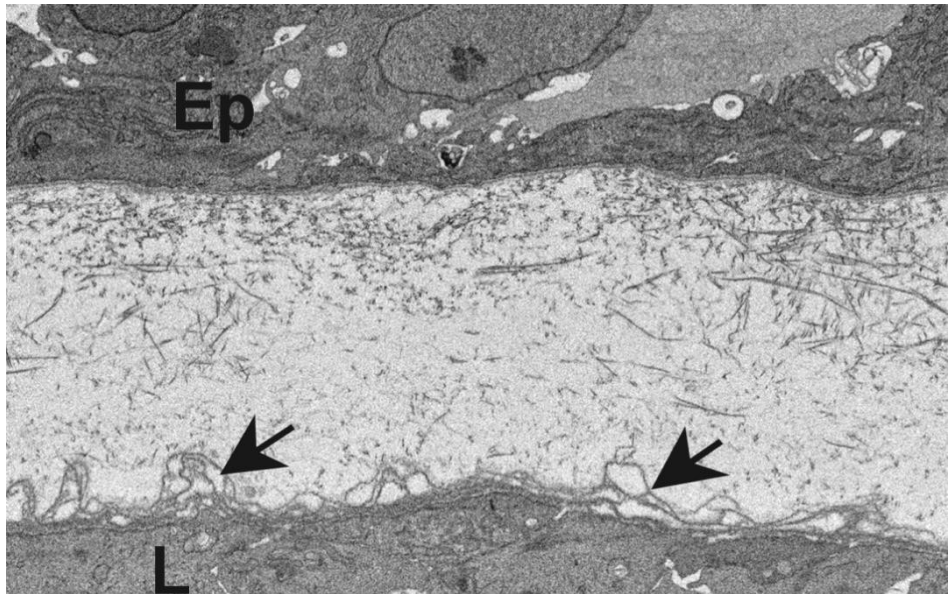

Figure 4: Transmission electron micrograph showing primary stroma at E4, separating epithelium (Ep) and lens surface (L), which exhibits a loose covering resembling basal lamina (arrows). Bar = 5 µm.

Later, between E4 and E5, neural crest cells destined to form the corneal endothelial monolayer were present, migrating across the posterior face of the primary stroma (Figure 5; Supplementary Video 2). These cells were characterised by the presence of innumerable cytoplasmic processes, which extended both into the overlying collagen fibrils of the primary stroma and proximally into the presumptive anterior chamber of the forming eye. SBF SEM enabled visualisation of abundant matrix cords evident from their protruding origins in the epithelial basement membrane and visible also within the vicinity of endothelial cells and their processes. Serial images, viewed using the volume viewer plugin for ImageJ/Fiji, clearly demonstrated the close proximity of endothelial cells and matrix cords, indicating, in some instances, envelopment of individual cords by cell processes (Figure 6).

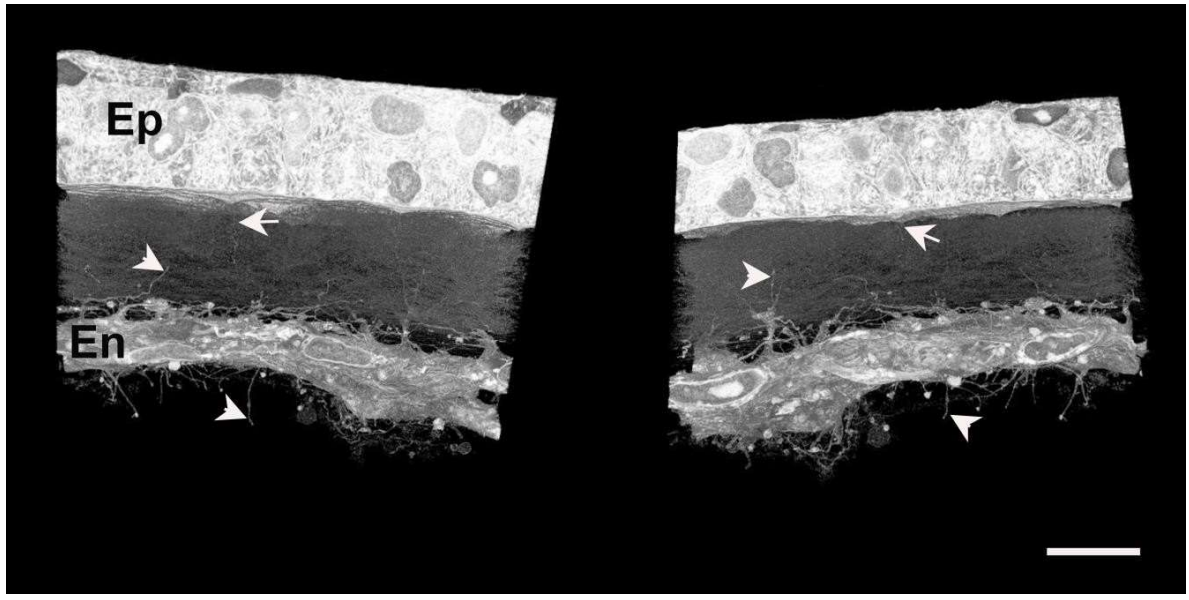

Figure 5: Reversed contrast images from a SBF SEM dataset of embryonic chick cornea at E4 +12h. Neural crest cells, migrating across the posterior surface of the primary stroma to form the corneal endothelium (En), exhibit multiple pseudopodial processes extending into the stroma and presumptive anterior chamber (arrowheads). Matrix cords emerge from protrusions in basement membrane of epithelium (Ep) and traverse the stroma (arrows) into the vicinity of migrating cell processes. Bar = 15 µm. Dataset viewable in Supplementary Video 2.

Figure 6: Three serial images at 100 nm intervals from an SBF SEM dataset of chick cornea at E5, viewed via the ImageJ/Fiji, Volume Viewer plugin, shows an endothelial cell (En) and cell process (arrowhead) in close contact with a matrix cord (arrow).

Discussion

Matrix structures in the developing avian cornea, which we have referred to as 'cords', have been recognised for many years, since some of the earliest studies of corneal development [1,13]. The consensus of opinion has generally been that they may have some role in the development of tissue transparency, perhaps linked to a mechanical function in preventing excessive swelling of the stroma as it hydrates and swells, before being populated by neural crest cells destined to become the corneal endothelium and keratocytes. On this basis, they could be viewed as being comparable to sutural fibres in the corneas of some other vertebrate species, such as reptiles and cartilaginous fish [14], which have similar origins in the epithelial basal lamina and traverse the stroma with a similar disto-proximal orientation.

Sutural fibres, however, appear morphologically far more substantial than their supposed counterparts in the avian cornea [15], which in some species, such as skates and rays, would seem consistent with a structural role in withstanding the swelling pressure of the stroma and preserving stromal thickness, in the absence of an endothelial layer [14,16]. In addition, whilst sutural fibres persist in the mature adult stoma, matrix cords have not been identified in the fully-formed avian cornea post-hatch. In our previous publication [8], we postulated that matrix cords may contribute to the development of tissue curvature, a vital property of the cornea allowing refraction of light for effective vision. This theory has yet to be tested. In the observations presented here, we show that matrix cords originate from early basal laminar connections between the corneal epithelium

and lens. An alternative explanation for a potential role therefore emerges, namely, that matrix cords have no function whatsoever and are merely vestiges of the separation of these two specialised structures. This possibility was first expressed by Winkler et al. [17] on the basis that the function of sutural fibres in restricting stroma swelling could be replaced in the avian stroma by the presence of lamellar branching, a feature not well developed in fish, amphibia and reptiles. In some datasets it is evident that an excess of frond-like basal lamina material seems to persist on the surface of the newly-independent lens. The attachments of matrix cords to protrusions in the basal lamina of the corneal epithelium are presumably more robust than those with the lens as they seem to detach from the latter more readily, early in E4. Although a contribution from mesenchymal cells cannot be conclusively excluded, we assume that the initial synthesis of matrix cords is primarily by the corneal epithelium, and that this continues, because they clearly elongate proximally as the stroma expands. Extension of laminal molecules by the epithelium into the stroma would imply their attachment to elements within the primary stroma, creating a tension along the length of the cord that would be consistent with the protruded deformations at points where they emanate from the corneal epithelial basement membrane, as is documented here (Figures 1, 3, and 5) and in our previous publication [8].

Close associations, in addition to those with matrix components, are clearly made also with inwardly migrating cells and we show here how presumptive endothelial cells, as well as keratocytes at later stages, appear to form attachments and interact with matrix cords. The concept that two-way interactions between extracellular matrix molecules and cells together influence tissue morphogenesis is now well accepted [18,19]. Cell adhesion and migration, and subsequent developmental events, may be influenced differentially by cues from both basement membranes and interstitial matrix, structurally and compositionally quite different substrates. If the composition of matrix cords resembles that of the basal lamina, a high potential for cell adhesion might be expected, and we are currently investigating this concept and the macromolecular identity of the matrix cords and surrounding matrix. Perhaps relevant to this line of thinking are the results of an immunohistochemical study of avian stroma by Fitch and associates [20], which described structures that the authors termed "strings". These resemble the matrix cords we report here, and were found to contain collagens type IV and V, and fibronectin, but not laminin and heparan sulphate proteoglycan, which are typical basement membrane constituents. The strings, however, were seen only after mesenchymal invasion and were presumed to be produced by stromal cells, whereas our identification of matrix cords precedes neural crest invasion and shows connectivity to the emerging, surface ectoderm-derived corneal epithelium and lens. Indeed, matrix cords are present in the early developing cornea even when neural crest cell migration is disturbed following removal of the lens at E3 [8], which supports the concept that they are synthesised by the corneal epithelium and/or lens rather than the invading presumptive keratocytes.

The first wave of neural crest cells entering the primary stroma, which are destined to form the corneal endothelium, appear to migrate as a discontinuous sheet along the diffuse posterior boundary of the primary stroma, expressing considerable membrane activity as evidenced by the abundance of elongate pseudopodial processes they express. These endothelial filopodia are quite different from the grossly extended, narrow projections of presumptive keratocytes, when they later deposit an aligned stromal extracellular matrix [7]. Endothelial precursor cells project processes both proximally into the presumptive anterior chamber and distally into the primary stroma, where they associate closely with both the collagen fibrils of the embryonic stroma and the population of matrix cords. Modelling studies have shown lead migrating neural crest cells respond primarily to long-range signals [21]. Of course, matrix cords might represent a vestigial remnant of very early stage ocular development, enduring after the once-connected presumptive lens and corneal epithelium have "moved away" from one another. But, it is not out of the question that cells pioneering the early avian cornea might derive guidance cues from both matrix cords and primary stroma, with an ability to detect both physical and compositional information, as well as themselves modifying the former by attachment and exertion of tractional forces on these components. This leads us to conjecture whether or not matrix cords might provide a conduit for interactions between mesenchymal cells and the corneal epithelium that could regulate cell behaviour via some form of mechanotransduction. Further investigation, naturally, would be required to examine this speculation.

Funding

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