Dynamic shape changes of ECM-producing cells drive morphogenesis of ball-and-socket joints in the fly leg

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SUMMARY
Animal body shape is framed by the skeleton, which is composed of extracellular matrix (ECM). Although how the body plan manifests in skeletal morphology has been studied intensively, cellular mechanisms that directly control skeletal ECM morphology remain elusive. In particular, how dynamic behaviors of ECM-secreting cells, such as shape changes and movements, contribute to ECM morphogenesis is unclear. Strict control of ECM morphology is crucial in the joints, where opposing sides of the skeleton must have precisely reciprocal shapes to fit each other. Here we found that, in the development of ball-and-socket joints in the Drosophila leg, the two sides of ECM form sequentially. We show that distinct cell populations produce the ‘ball’ and the ‘socket’, and that these cells undergo extensive shape changes while depositing ECM. We propose that shape changes of ECM-producing cells enable the sequential ECM formation to allow the morphological coupling of adjacent components. Our results highlight the importance of dynamic cell behaviors in precise shaping of skeletal ECM architecture.

KEY WORDS: Joint, Ball-and-socket, Cuticle, Chitin, Epidermis, Cell shape change, Leg, Drosophila

INTRODUCTION
Regulation of the body shape has been a central issue in developmental and evolutionary biology. The skeleton provides the framework of animal body shape. In vertebrates, bones act as the inner core of the body, although there are some skeletal elements that appear and function on the exterior, such as teeth, beaks, scales and antlers. Skeletons of invertebrates are predominantly external: cuticles on the body surface. Intensive studies have revealed that genetically programmed body plans ultimately dictate skeletal patterns. However, cellular mechanisms that directly control skeletal morphology downstream of those genetic programs remain unknown.

The skeleton is composed of extracellular matrix (ECM), consisting of molecules secreted from cells. Bones comprise collagen and proteins secreted by osteoblasts (Hall, 2005), whereas invertebrate cuticles consist of proteins and chitin, a long-chain sugar polymer, released by the underlying epidermal cells (Chapman, 1998). The final morphology of skeletal ECM should reflect not only the terminal arrangement of ECM-secreting cells but also the history of how their number, position, shape and activity have changed during the entire ECM formation period. To understand the cellular mechanisms for skeletal morphogenesis, we therefore need to look at the dynamic behaviors of those cells and to determine how they relate to ECM morphology.

Stringent regulation of ECM morphology is particularly important and prominent in the joints, which are the flexible parts of the skeleton that permit movement. The human skeleton possesses joints of various configurations, such as ball-and-socket, hinge, saddle and pivot (Standring et al., 2005). In each configuration, opposing sides of the ECM must be shaped in a precisely reciprocal manner to form a tightly interlocking structure for efficient and controlled motion. Although the vertebrate synovial joints have long received attention for their medical importance, the mechanisms by which they actually form during embryogenesis have been poorly understood (Khan et al., 2007; Pacifici et al., 2005).

The exoskeletal system offers some technical advantages to address this issue. The epidermis consists of a single, continuous sheet of epithelial cells that are polarized along the apicobasal axis: the apical surfaces of the cells face the body surface and secrete cuticle. The epidermis maintains its integrity and apicobasal polarity and continues to adhere to the cuticle without mixing with it (Chapman, 1998). Within the epidermal sheet, cell identities are specified by two-dimensional positional cues (Cohen, 1993; Payre, 2004). This specification process is separated in time from the later differentiation, when cells actually make specific parts of the cuticle (Fristrom and Fristrom, 1993). Moreover, morphogenesis of the adult cuticle appears to be completed before the muscles start to contract just prior to eclosion. All of these features make the exoskeleton a promising system for studying the dynamic behaviors of cells during ECM formation and how they are linked to the final ECM morphology.

We focus on the ball-and-socket joints in the tarsus of the Drosophila leg. How their positions are determined in the leg primordium before pupariation has been studied extensively (Kojima, 2004), and the central role of the Notch signaling pathway therein has been demonstrated (de Celis et al., 1998; Rauskolb and Irvine, 1999). The subsequent folding (Manjon et al., 2007), unfolding and re-constriction (Fristrom and Fristrom, 1993; Mirth and Akam, 2002) of the epidermis in the joint regions have also been described. By contrast, the way in which the joint cuticles develop afterwards and how the cells behave during that period are largely unknown. As it takes more than three days from...
specification of the joint regions to the completion of the joint cuticle architecture, the essence of ECM morphogenesis must lie in how cells act during this period.

In this study, we describe the development of the ball and the socket cuticles and the concomitant cellular morphogenesis, which occur long after cell specification. Through chronological observation of cellular and cuticular architectures, we reveal that the ball and the socket cuticles develop in a sequential manner, and that the two parts are formed by distinct cell populations that undergo extensive shape changes while secreting cuticle. We propose that the shape changes of ECM-producing cells result in the sequential formation of the ball and the socket, permitting their morphological coupling in a ‘mold-and-cast’ manner.

**MATERIALS AND METHODS**

**Fly strains and confocal microscopy**

Fly strains used were: sqh-GFP-Moesin (Kiehart et al., 2000), Myosin Regulatory Light Chain (MRLC)-GFP (Royou et al., 2004), UAS-Serp(CBD)GFP (Luschinig et al., 2006), UAS-GFP-TTras (Kato et al., 2004), UAS-krotzkopf verkehr (kkv) RNAi (Dietzl et al., 2007), Distal-lessRNAi (Dl-GAL4) (Calleja et al., 1996), big bristleRNAi (bbi-GAL4) and fringeRNAi (fringe-GAL4) (Hayashi et al., 2002), neuralizedGFP (neur-GAL4) (Bellaiche et al., 2001), UAS-KO, ubi-KOnls (Kaido et al., 2009), ubi-GFPs65Tls, UAS-GFP.S65T and dautelous (da)-GAL4 (Bloomington Stock Center). sqh-GFP-Moesin or MRLC-GFP was used as wild type unless otherwise noted.

**Confocal microscopy and leg staining**

Our method is based on that described by Mirth and Akam (Mirth and Akam, 2002). Briefly, pupal thoraces were dissected in PBS and transferred to 4% paraformaldehyde in PBS. After removal of pupal cuticles, the legs were fixed for 20-30 minutes at room temperature and washed with PBST. The following probes and dilutions were used: mouse anti-chitin antibody or fluorescent probes and washed with PBST before mounting.

**Electron microscopy**

Thoraces were dissected from pupae in PBS, and legs were immediately embedded in 1% agarose and subjected to post-fixation with 1% OsO4 in 0.1 M cacodylate buffer for 8 hours on ice, and then to en bloc staining in 0.5% uranyl acetate aqueous solution overnight at room temperature. Specimens were dehydrated in ethanol and embedded in Poly/Bed812 (Epon 812, Polysciences). Sections stained with uranyl acetate and lead citrate were viewed in a JEOL JEM-1010 transmission electron microscope at 100 kV.

**Verification of kkv RNAi**

In order to verify the effect of RNAi against kkv, progeny from the crossing of males with two copies of UAS-kkv RNAi and da-GAL4 females were stained with Fluostain (Moussian et al., 2005) or processed for cuticle preparation with or without the vitelline membrane in Hoyer’s medium (Sullivan et al., 2000).

The effect of kkv RNAi on chitin accumulation in the ball cuticle was quantified as follows (see Fig. S3A-C in the supplementary material). Legs from RNAi (bib-GAL4/UAS-kkv RNAi; UAS-GFP-TTras/+), sibling control (CyO/UAS-kkv RNAi; UAS-GFP-TTras/+), or progeny from the crossing (kkv RNAi; UAS-GFP-TTras/+) pupae (at least four for each genotype) were pooled and divided into two batches: one for Fluostain staining and the other for negative control for background measurement. Confocal images were taken with an Olympus FV1000, ×60 water immersion lens, NA=1.2, LD405 nm laser (50 mW) at 0.1% intensity, PMT 550 V, aperture 120 μm and fluorescence intensity was measured using ImageJ (see Fig. S3A in the supplementary material).

**Time-lapse imaging**

Pupae were removed from pupal cases and placed on glass-bottomed dishes. Time-lapse images were taken with an Olympus FV1000 confocal scanning microscope, and processed using iSEMS (Kato and Hayashi, 2008).

**Electron microscopy**

Thoraces were dissected from pupae in PBS, and legs were immediately transferred to fixation buffer (2.5% glutaraldehyde, 2% formaldehyde, 0.1 M cacodylate buffer). Pelopon cuticles were removed in the buffer, and legs were incubated for >2 hours at room temperature. Fixed legs were embedded in 1% agarose and subjected to post-fixation with 1% OsO4 in 0.1 M cacodylate buffer for 8 hours on ice, and then to en bloc staining in 0.5% uranyl acetate aqueous solution overnight at room temperature. Samples were subsequently dehydrated in ethanol and embedded in Poly/Bed812 (Epon 812, Polysciences). Sections stained with uranyl acetate and lead citrate were viewed in a JEOL JEM-1010 transmission electron microscope at 100 kV.

**Fig. 1. Mature joint morphology.** (A) Cuticle preparation of the adult leg (tarsus). ta1-ta5, tarsomeres 1-5. Arrows indicate the joints focused on in this study; arrowhead indicates the joint between ta4 and 5. (B-D) Mature structure of the three proximal inter-tarsomere joints (arrows in A). (B-B') Electron micrograph of the mature joint. Boxed areas in B are enlarged in B' and B''. (C-C') Confocal microscope image of the mature joint. (C') Differential interference contrast (DIC) image. (C) The ball and socket cuticles are labeled by a chitin-binding probe (magenta). GFP-Moesin labels actin enrichment at the cell cortices (green). (D) Serp(CBD)GFP (green) is overexpressed using Dl-GAL4 in the mature joint. Cuticles are labeled by a chitin-binding probe (magenta). (E-E') The joint between ta4 and ta5, labeled as in C and C'. Dorsal is up and distal is to the right in this and all subsequent figures unless otherwise noted. so, socket cuticle; lu, lubricant matrix. Scale bars: 2 μm in B; 1 μm in B', B''; 5 μm in C-E'.
Nikkomycin injection

Pupae at 40-45 hours APF (a few hours prior to the onset of cuticle formation) were sterilized by soaking in 70% ethanol and placed on plastic dishes with adhesive tape. Nikkomycin Z (Sigma-Aldrich) was dissolved in water (2.5 mg/ml) and 0.5~1.0 μl of the solution was injected into the thoracic region of pupae by piercing a hole in the pupal case with a glass needle. Water injection was used as a control. Approximately 24 hours after injection at 25°C, the legs were prepared for viewing under a confocal microscope. Cuticle preparations in Gary’s Magic Mountant (Roberts, 1986) were made from pupae incubated for more than 48 hours after injection.

RESULTS

Mature joint morphology

The tarsus comprises five segments (tarsomeres), separated by joints (Fig. 1A). On the dorsal side of the inter-tarsomere joints, the cuticle has the ball-and-socket architecture (Fig. 1). The joint between tarsomeres 4 and 5 had a slightly different ball-and-socket morphology (Fig. 1E), with a smaller ball and a thicker socket than the other three (Fig. 1C,D). In this study, we focused on the three proximal inter-tarsomere joints, which develop simultaneously. Both the ball and the socket were labeled by a chitin-binding probe (Fig. 1C,D). The ball-and-socket structure was tucked under the ridge, on which the socket cuticle spreads (Fig. 1B,C). On the ventral aspect of the ball, a distally oriented process, which we named the ‘lip’, grew into the epithelium (Fig. 1B,C). Electron microscopy (EM) revealed a strikingly regular stratification of the ball cuticle, consisting of 70-80 layers of approximately 100-150 nm thickness (the variation might depend on the angles of thin sections; Fig. 1B). It also uncovered the matrix that filled the space between the ball and the socket, sealed by the membranous cuticle (Fig. 1B). A secreted form of GFP [Serp(CBD)GFP (Luschnig et al., 2006)] was enriched in this matrix when it was expressed by underlying cells (Fig. 1D). This matrix was labeled poorly with the chitin-binding probe and we speculate that it acts as a lubricant in the moving adult legs.

Sequential development of the joint

The leg primordium is composed of an epithelium with deep segmental folds during the prepupal period (de Celis et al., 1998) that then elongate to become a straight and smooth cylinder by the prepupa-pupa transition (apoplysis) (Fristrom and Fristrom, 1993; Mirth and Akam, 2002). At this stage (~20 hours APF, Fig. 2A), cells in the joint region were aligned along the circumference (Fig. 2A'). They underwent apical constriction (not shown), resulting in the constriction of the leg in the joint regions (Fig. 2B,B'). When the entire leg subsequently became thinner (Fig. 2C), the cells in the joint region remained neatly aligned and constricted (Fig. 2C'). Cells on the dorsal side of the joint constricted further and infolded proximally (Fig. 2D-D'). By ~45 hours APF, the furrow grew deeper and resulted in a deep cavity (Fig. 2E-E').

Cuticle deposition in the joint region began at 45-48 hours APF when the chitin-binding probe revealed signals covering both the dorsal and the ventral sides of the cavity (Fig. 3A-A'). The cuticle in the deepest part (bottom) of the cavity then thickened to form the ball cuticle (Fig. 3B-D'). Electron micrographs indicated that layers of consistent thickness were added successively to the ball cuticles (Fig. 4B,C). At 63-66 hours APF, the ball cuticle reached its full size.

At this same stage, the socket cuticle was formed only on the dorsal side of the cavity (Fig. 3D,D'; Fig. 4C-C'). The socket cuticle then expanded gradually in the dorsal-to-ventral direction towards the bottom of the cavity (Fig. 3E-E'), until eventually covering the entire cavity at ~75 hours APF (Fig. 3F-F', Fig. 4D). During this time, the ‘lip’ of the ball cuticle also developed (Fig. 3E-F'; Fig. 4D).

Thus, the ball and the socket are produced in a sequential manner: the socket cuticle develops throughout the cavity only after the ball has reached its full size. We propose that the serial formation of the ball and the socket serves as ‘mold casting’, which permits their morphological coupling (see Discussion).

It has been reported that most of the final cell divisions are complete by about 30 hours APF (Fristrom and Fristrom, 1993), suggesting that the sequential formation of the ball and the socket cuticles shown in Fig. 3 progresses without cell proliferation. We next addressed the cellular mechanism underlying such sequentiality.

Sites of ball/socket production revealed by electron microscopy

In order to study the spatio-temporal pattern of cuticle secretion at the cellular level, we used EM analysis to monitor the appearance of the histological structure called plasma membrane plaques.
PMPs are electron-dense regions of the plasma membrane located at the tips of microvilli in contact with newly synthesized cuticles of various insects (Chapman, 1998; Locke and Huie, 1979), and are regarded as the sites of chitin microfibril release and/or cuticle-cell adhesion.

At the onset of ball formation, the apical surfaces of the cells surrounding the ball cuticle contained many PMPs (Fig. 4A,A’). The PMPs were in contact with fibrous materials, presumably chitin microfibrils, arranged parallel to the ball cuticle (Fig. 4A’). Thus, these cells contacted the ball cuticle, and were probably adding chitin microfibrils to it. The thin and scattered socket cuticle was evenly associated with PMPs of the cells on the dorsal side (Fig. 4A).

![Fig. 3. Sequential formation of the ball and the socket cuticles.](image)

Sequence of ball-and-socket cuticle formation observed by confocal microscopy. (A-F) DIC images. (A’-F’) Cuticles are stained with a chitin-binding probe (magenta), and myosin or actin enrichment at cell cortices is labeled by MRLC-GFP in B’-E’, or by GFP-Moe in F’ (green). (A’-F’) Schematic representations. (A-A’) At 45-48 hours APF, cuticles are detected on the dorsal (arrow) and ventral (asterisk) sides of the cavity. (B-B’) By 52-55 hours APF, the ball cuticle on the ventral side of the cavity has thickened (B’, asterisk). An arrow marks the edge of the socket cuticle. (C-C’) The ball cuticle has further enlarged (asterisk) by 55-63 hours APF. The socket cuticle has not yet extended significantly (arrow). (D-D’) At 63-66 hours APF, the ball has reached its full size (asterisk). The socket is detected only on the dorsal side of the cavity (arrow). (E-E’) By 70-72 hours APF, the socket is extending along the cavity into the lateral side of the ball (arrow) and the ‘lip’ is developing (arrowhead). (F-F’) By 72-75 hours APF, the socket has extended to the ventral side of the ball (arrow) and the lip has fully formed (arrowhead). Scale bar: 5 μm.

![Fig. 4. Distribution of plasma membrane plaques (PMPs) at different stages of cuticle formation.](image)

(A-D) Electron micrographs of joints at different stages of cuticle formation. Yellow arrows indicate PMPs. (A) At 55 hours APF, the ball cuticle is thickening (asterisk) and the socket cuticle is composed of thin and scattered pieces (bracket). White arrow indicates the edge of the socket. (A’) Magnification of the boxed area in A. Brackets indicate presumptive chitin microfibrils. (B) At 58 hours APF, the ball continues to enlarge (asterisk). The edge of the socket is indicated by white arrow. (B’,B”) Enlargement of the boxed regions in B. White arrows indicate pieces of the socket cuticle. (C) By 65 hours APF, the ball has reached its full size (asterisk). The lip has not yet formed in this sample. (C’) Magnification of the boxed region in C showing that the socket cuticle is associated with PMPs. (D) By 72 hours APF, the socket has formed throughout the cavity, reaching the ventral side of the ball cuticle (edge indicated by arrow). (E) Changes in the distribution of PMPs contacting the ball cuticle and those contacting the socket. Scale bars: 1 μm in A,B,C,D; 500 nm in A’,B’,B”,C’,C”.
During its growth, the ball cuticle continued to be surrounded by PMPs (Fig. 4B,B'). However, when it had fully grown, the PMPs were relatively sparse on its lateral side (Fig. 4C,C'). The socket cuticle, which was still thin and scattered at this point, continued to be associated with PMPs (Fig. 4B,B',C,C'). When the socket cuticle formed in the bottom of the cavity, it was uniformly lined with PMPs (Fig. 4D). PMPs were also found enriched along the cuticle formed in the bottom of the cavity, which was still thin and scattered at this point, continued to be associated with PMPs (Fig. 4B,B').

These results from electron microscopy experiments are summarized in Fig. 4E. Notably, the positions of the ball-contacting area (blue) and the socket-contacting area (red), which presumably comprise ball-producing activity and socket-producing activity, respectively, shift extensively along the cavity during cuticle formation. If cells remain stationary, the ball-producing and socket-producing activities must sequentially progress across the cells, in a wave-like fashion. If, conversely, the ball-producing cells and the socket-producing cells retain their respective identities as separate populations, they must shift their apical surfaces along the cavity by cell movement or shape changes. With this in mind, we next examined the cell dynamics during cuticle formation.

Extensive cell shape changes in the joint region during cuticle formation

We sought to label subpopulations of cells within the joint region using the GAL4-UAS method (Brand and Perrimon, 1993) and to monitor their locations and shapes at various stages. Among GAL4 drivers inserted near the genes reported to show segmental expression in the tarsus, we found three drivers which were expressed in different but overlapping populations of cells in the joint region: big brain (bib)-GAL4, neuralized (neur)-GAL4 and fringe (fng)-GAL4 (de Celis et al., 1998; Mirth and Akam, 2002; Rauskolb and Irvine, 1999; Shirai et al., 2007). bib and neur were expressed in the deep region of the cavity, flanked by fng expression in cells projecting into the ridge (see Fig. 1C') and cells on the ventral side of the cavity (Fig. 5D,G,J). Simultaneous detection of bib (bib-GAL4 and UAS-GFP) and neur (neur-lacZns) revealed that bib was expressed distal to neur, with an overlap of about two cells (Fig. 5A-A').

GAL4-expressing cells were outlined by a membrane-bound GFP (Fig. 5B-J). Shortly after the onset of ball formation, the bib-expressing cells were in contact with the ball cuticle (Fig. 5B). The
expression of neur appeared to overlap largely with bib-expressing cells and to extend dorsally to them (Fig. 5C; consistent with Fig. 5A-A’). bib and neur expression domains were four to six cells wide along the anteroposterior axis (data not shown).

When the ball had fully grown, the apical surfaces of bib-expressing cells came into contact with the ventral side of the ball (Fig. 5E), and then the apical surfaces further shifted distally eventually contacting the newly formed lip (Fig. 5H). Consistently, the apices of the ventral portion of neur-expressing cells (co-expressing bib) relocated to the ventral side of the ball (Fig. 5F,I), and the apical surfaces of their dorsal subsets moved with the edge of the extending socket, until covering the ventral part of the socket (Fig. 5F,I). Meanwhile, the dorsal fng-expressing cells expanded their apical surfaces (Fig. 5G,J), and when the socket cuticle formed at the bottom of the cavity, they covered a large dorsal portion of the socket cuticle (Fig. 5J). A similar observation was made on legs in which all of the cells in the joint region were labeled by Dil-GAL4 (data not shown). Furthermore, tracing the cell shapes in electron micrographs confirmed extensive changes in the morphology of the cell contacting the ball and socket cuticles (see Fig. S1 in the supplementary material).

We also took a live-imaging approach in order to directly monitor the dynamic behaviors of bib- and neur-expressing cells. Pupal legs were doubly labeled with fluorescent proteins expressed by bib-GAL4 or neur-GAL4, and ubiquitously expressed nuclear localized markers (see Movies 1-3 in the supplementary material) and imaged over 5 hours. The movies allowed us to continuously trace the bib-GAL4- and neur-GAL4-expressing cells, which moved their apical surfaces extensively along the cavity.

Fig. 5K shows a summary of the cell shape changes, overlaid on the distribution of PMPs at respective stages. Notably, the movement of the apical surfaces of the cells continuously paralleled the distribution changes of PMPs. This parallelism is consistent with the model that the ball-producing cells and the socket-producing cells retain their respective identities as separate populations (see Discussion).

Identification of ball-producing and socket-producing cell populations

To identify which cuticular elements are produced by each of the bib-, neur- and fng-expressing cells, we attempted to interfere with cuticle production in a cell-specific manner using transgenic RNAi constructs of kroetzkopf verkehrte (kkv), encoding Chitin Synthase 1. The UAS-kkv RNAi strains used here caused defects phenocopying those of kkv mutants: attenuation of chitin accumulation in the tracheal lumen and its failure to expand uniformly (Devine et al., 2005; Tonnig et al., 2005), deformation of the head skeleton, and reduction of cuticle strength (Moussian et al., 2005; Ostrowski et al., 2002) (see Fig. S2 in the supplementary material). Measurement of the chitin-staining dye Fluostain demonstrated that kkv RNAi robustly reduced the amount of chitin in the ball cuticle (see Fig. S3A-C in the supplementary material).

As a positive control for chitin synthesis attenuation, we injected Nikkomycin Z, a GlcNAc analog that competitively inhibits chitin synthase activity (Tellam et al., 2000), into the pupal thoraces before the onset of cuticle formation. Fig. 6B shows the typical morphology of the joint cuticle that developed in wild-type pupae injected with Nikkomycin. The cuticle consisted of multiple amorphous bulges: one relatively large bulge on the ventral side of the cavity that presumably derived from the ball, and a few smaller ones on the lateral and dorsal sides that probably correspond to the socket. Thus, the morphology of the entire ball-and-socket cuticle was severely disrupted by the global attenuation of chitin synthesis.

We next used bib- and neur-GAL4 drivers for knock-down experiments. Activities of those drivers were approximately the same, as assessed by the measurement of GFP signals expressed by those drivers (see Fig. S3D in the supplementary material). When kkv RNAi was induced by bib-GAL4, the morphology of the ball cuticle was disrupted as severely as seen in Nikkomycin-injected samples (Fig. 6C; see also Fig. S3E in the supplementary material). The socket formation was also inhibited, although we could often discern the dorsal portion of the socket. Conversely, when neur-GAL4 was used, the ball cuticle was largely intact, whereas the ventral part of the socket was missing (Fig. 6D; see also Fig. S3E in the supplementary material). Thus, neur>kkv RNAi preferentially affected the socket rather than the ball, whereas bib>kkv RNAi strongly affected both (Fig. 6C,D; see also Fig. S3E in supplementary material). The overlap in their phenotypes in the socket cuticle is likely to reflect the overlap of bib and neur expression (Fig. 5A-A’; see Discussion). Use of fng-GAL4 caused yet another phenotype: the ball morphology was largely normal, whereas the socket cuticle had a bloated appearance and a few bulges (Fig. 6E).

Altogether, attenuation of chitin synthesis in specific cell populations differentially affected both the ball and the socket, and the ball-producing activity and the socket-producing activity were mapped to different cell populations along the proximodistal axis,
as shown in Fig. 6F. Importantly, this map is consistent with the part of the cuticle that each cell population continues to associate with during cuticle formation (Fig. 5K; see Discussion).

We also attempted to locate the lubricant-producing activity. As shown in Fig. 1D, Serp(CBD)GFP was enriched in the lubricant when it was expressed by Dll-GAL4. When Dll-GAL4 was replaced with either bib- or neur- or fng-GAL4, Serp(CBD)GFP still localized in the lubricant (see Fig. S4 in the supplementary material), suggesting that the lubricant-secreting activity is distributed rather broadly in the joint region. Alternatively, it could be that proteins secreted by cells outside of this region are transported into the lubricant.

**Shape change of cuticle-secreting cells proceeds even when cuticle morphology is severely disrupted**

As shown above, the cells progressively relocated their apical surfaces in the proximal-to-distal direction (Fig. 5; see also Movies 1-3 in the supplementary material), and this was accompanied by polarized growth of the cuticle in the same direction: stratification of the ball progressed from dorsal to ventral, where the apical and the lip both elongated from proximal to distal. Interestingly, the apical surfaces of the ball-producing cells moved together with the lip, whereas those of the socket-producing cells followed the edge of the socket cuticle (Fig. 5). These observations prompted us to test whether the cuticle acts as a structural guidance cue informing the cells of the direction or the destination of movement.

We used Nikkomycin treatment, which causes severe disruption of the morphology and continuity of the entire joint cuticles (Fig. 6B), to determine whether the cuticle has a guidance role. Nikkomycin was injected at the onset of cuticle formation into pupae in which bib- or fng-expressing cells were labeled by membrane-bound GFP, and the cell and ECM morphologies were examined after 24 hours, when cell shape changes would have been completed under normal conditions (cf. Fig. 5G-I). Typically, we saw that the ball developed as an amorphous bulb without the ‘lip’, and the socket disintegrated into multiple small bulges (Fig. 6B; Fig. 7A,B). In severe cases, the ball even appeared to be fragmented into small pieces (Fig. 7C,D).

To our surprise, the overall shapes of bib- and fng-expressing cells in Nikkomycin-injected pupae were comparable to those in controls. The apical domains of bib-expressing cells had relocated to the ventral side of the cavity, where they would have normally contacted the lip (Fig. 7A,C). Thus, the movement of the apices of ball-producing cells appears to be capable of orienting correctly in the absence of the normal cuticular morphology, including the lip. Likewise, the apical surfaces of the dorsal fng-expressing cells extended ventrally out of the ridge (Fig. 7B,D), similarly to how they move in standard conditions. The shape changes of neur-expressing cells in Nikkomycin-injected pupae were also comparable to those in the normal situation (data not shown). The fact that all of the cells relocated their apical surfaces in the correct direction and to the correct destination, even when the cuticle morphology was severely disrupted, suggests that the normal shape and continuity of the cuticle is dispensable for guiding the migration of the apical surface of cuticle-secreting cells.

We also noted that the apical surfaces of socket-producing cells expanded so as to wrap around the misshapen or fragmented ball cuticle (Fig. 7B,D), indicating that the final shapes of socket-producing cells respected the morphology of the remaining ball cuticle. This local adjustment of cell shape might reflect the effect of remaining cuticle components. Because the Nikkomycin treatment did not completely eliminate the cuticle, it is possible that the cuticle might play a permissive role in cell movement; for example, by acting as a substratum for migration.

**DISCUSSION**

In this study, we revealed that the ball and the socket cuticles develop sequentially. We have shown that the ball-producing activity and the socket-producing activity are allocated to distinct cell populations, and have found that shape changes of these cells that occur simultaneously with their cuticle-secreting activities result in the interlocking ball-and-socket structure. As the ball cuticle builds up, concurrent cell shape changes drive the apical domains of ball-producing cells out of the cavity and bring in the apical domains of the socket-producing cells, resulting in close enwrapping of the ball by the latter cell population. Accordingly, the shape of the resulting socket cuticle conforms to that of the ball. Synchronization between ECM formation and dynamic relocation of the cell surfaces that mediate it thus underlies the building of the complex ECM structure.

**Mapping of ball-producing cells and socket-producing cells**

The map of ball-producing and socket-producing cells shown in Fig. 6F best summarizes the results of kkv RNAi, and is consistent with the result indicating their continuous association with respective parts of the cuticle during their formation (Fig. 5K). The ball morphology was severely disrupted by bib>kkv RNAi but not by neur>kkv RNAi, indicating that the ball-producing activity is restricted to the distal subset of bib-expressing cells that do not significantly express neur. Consistently, these cells are in constant contact with the ball cuticle throughout its formation. The cuticle phenotype of neur>kkv RNAi shows that neur-expressing cells are
responsible for forming the ventral part of the socket cuticle, with which they continue to associate. Likewise, fng-expressing cells contribute to the formation of the remainder of the socket. Partial disruption of the socket by bib-kkv RNAi (Fig. 6C; see Fig. S3E in the supplementary material) should be, to some extent, due to direct blocking of socket production in cells co-expressing bib and neur. Additionally, the impairment of ball formation might somehow interfere with socket formation. Occasional deformation of the ball by neur-kkv RNAi (see Fig. S3E in the supplementary material) might be caused by marginal expression of neur in the presumptive ball-producing cells, which is barely detectable in Fig. 5A-A’.

Role of dynamic cell behaviors in regulating ECM morphology

Patterns of ECM-producing tissues do play a major role in the regulation of ECM morphology. Previous studies have unraveled how global positional information affects skeletal patterns through the regulation of specification, differentiation and proliferation of ECM-producing cells (Hall, 2005; Payre, 2004). There, the morphology of ECM was assumed to be synonymous with that of the cells or tissues that secrete it. Our present study illustrates that the skeletal morphology reflects not only the pattern of those cells at one point in time, but also the history of their dynamic behaviors during ECM formation. Secreted apically by the epidermis, the cuticle is monolayered in most parts. In the joints, however, relocation of the secretory surfaces enables formation of a cuticle beneath a pre-formed layer. Cell motility thus allows a tissue of simple configuration to build a complex and essential three-dimensional ECM structure. We envision that movements of ECM-secreting cells probably play important roles in ECM morphogenesis in other systems, especially in formation or adjustment of intricate skeletal structures.

Mechanism for cell shape changes

The morphology of the cuticle, as well as how it develops, correlated well with cell shape changes (Fig. 5K). This suggests either that the cell-shape changes govern the morphology of the cuticle, and/or vice versa. We found that the movement of the apical surfaces of the cells was correctly oriented even when the shape of the cuticle was disrupted (Fig. 7), indicating that the morphogenesis of the ball-and-socket cuticle is primarily controlled by the way the cells change their shapes as they deposit the cuticle. How do the cells know which way to move? In other words, what is the molecular mechanism that mediates global proximodistal polarity of the leg to direct cell movement? In mutants of well-known planar cell polarity genes, such as frizzled, dishevelled and prickle, extra joints of reverse proximodistal polarity are formed (Held et al., 1986). Nonetheless, the ball-and-socket structure of individual joints remains largely intact (data not shown), indicating that cell shape changes are correctly guided by a mechanism other than this pathway. Analysis and local disruption of cytoskeletal architecture in the joint region could help us to answer this question.

As mentioned earlier, our results do not rule out the possibility that the cuticle plays a permissive role in cell movements. The ECM generally affects cell shape and motility (Alberts et al., 2002), and chitin-based ECM has been shown to regulate epithelial morphogenesis in some Drosophila tissues (Devine et al., 2005; Moussian et al., 2005; Tonning et al., 2005). Whether the cuticle provides a permissive environment for cell shape changes in the joint is an important issue to address in future work.

‘Mold casting’ – a general mechanism for joint development?

The formation of reciprocally shaped interfaces is vital for the sake of joint function. The serial progression of ball-and-socket morphogenesis shown here can be compared to ‘mold casting’: (1) the ball enlarges rapidly through stratification, and the cavity expands to accommodate it; and (2) the enlarged cavity then serves as the ‘mold’ along which the socket cuticle is formed. Hence, the shape of the ball is transmitted to the socket (the ‘cast’). Whether or not this model also applies to vertebrate synovial joints is an intriguing question. Pacifci et al. speculated that, in the chick digit joints, chondrogenic cell differentiation on the distal side might promote its expansion to become convex; at the same time, proliferation of peripheral cells on the proximal side might permit them to grow and wrap themselves around the distal side, thereby becoming concave (Pacifci et al., 2005). If this were the case, that model can be regarded as a modified version of our ball-and-socket morphogenesis, one side fitting to the other through cell proliferation instead of cell shape changes. It will then become important to study how cells and ECM collectively undergo morphogenesis in other types of joints and in other species. Unraveling similarities and differences in the modes of joint development would be crucial in a medical sense as well, for understanding various joint pathologies and designing therapies to treat them.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Ball-and-socket joint morphogenesis

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