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Early patterning of the spider embryo: a cluster of mesenchymal cells at the cumulus produces Dpp signals received by germ disc epithelial cells

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SUMMARY

In early embryogenesis of spiders, the cumulus is characteristically observed as a cellular thickening that arises from the center of the germ disc and moves centrifugally. This cumulus movement breaks the radial symmetry of the germ disc morphology, correlating with the development of the dorsal region of the embryo. Classical experiments on spider embryos have shown that a cumulus has the capacity to induce a secondary axis when transplanted ectopically. In this study, we have examined the house spider, *Achaearanea tepidariorum*, on the basis of knowledge from *Drosophila* to characterize the cumulus at the cellular and molecular level. In the cumulus, a cluster of about 10 mesenchymal cells, designated the cumulus mesenchymal (CM) cells, is situated beneath the epithelium, where the CM cells migrate to the rim of the germ disc. Germ disc epithelial cells near the migrating CM cells extend cytoneme-like projections from their basal side onto the surface of the CM cells. Molecular cloning and whole-mount in situ hybridization showed that the CM cells expressed a spider homolog of *Drosophila decapentaplegic* (*dpp*), which encodes a secreted protein that functions as a dorsal morphogen in the *Drosophila* embryo. Furthermore, the spider Dpp signal appeared to induce graded levels of the phosphorylated Mothers against dpp (Mad) protein in the nuclei of germ disc epithelial cells. Adding data from spider homologs of *fork head*, *orthodenticle* and *caudal*, we suggest that, in contrast to the *Drosophila* embryo, the progressive mesenchymal-epithelial cell interactions involving the Dpp-Mad signaling cascade generate dorsoventral polarity in accordance with the anteroposterior axis formation in the spider embryo. Our findings support the idea that the cumulus plays a central role in the axial pattern formation of the spider embryo.

Movie and supplemental figure available online

Key words: Spider, Chelicerate, Embryogenesis, Cumulus, *dpp*/BMP2/BMP4, *fork head*/HNF3, *otd/otx*, *cad*/*cdx*, Mad/Smad, Body axis formation

INTRODUCTION

Establishment of dorsoventral (DV) polarity is essential for bilaterians to shape their body. In the development of many bilaterians, the DV axis, which is orthogonal to the anteroposterior (AP) axis, becomes morphologically apparent through a chain of asymmetric cell movements and behaviors. In the development of the chelicerate spider embryo (Montgomery, 1909; Holm, 1940; Holm, 1952; Seitz, 1966), the first morphological DV asymmetry can be recognized by the formation and movement of the cumulus, which appears from the center of the symmetrical germ disc and moves straight to the rim. The cumulus is a cellular thickening that makes a bulge on the surface of the germ disc. The functional importance of the cumulus in early patterning of the spider embryo was demonstrated by Holm (Holm, 1952), who used embryos of the spider, *Agelena labyrinthica*. Extirpation of the cumulus produced abnormal embryos that lacked the dorsal area. By contrast, transplantation of a part of the cumulus to ectopic sites frequently resulted in twined embryos. Based on these data, Holm suggested that the cumulus, like the dorsal lip in amphibians, is an organizing center for the axial pattern formation of the spider embryo. Despite this fascinating idea, however, the cumulus has been poorly characterized in a cellular and molecular context.

Cellular and molecular mechanisms that control early patterning have been most extensively studied for the insect, *Drosophila melanogaster*. In the early *Drosophila* embryo, AP and DV asymmetries pre-exist as localized maternal components, which give rise to a region-specific zygotic expression of genes (St Johnston and Nüsslein-Volhard, 1992; Driever, 1993; Chasan and Anderson, 1993). The DV polarity of the *Drosophila* embryo comes from a regulated nuclear localization of the maternal transcription factor Dorsal. A gradient of nuclear Dorsal concentration along the DV axis subdivides the embryo into a series of domains with different fates: amnioserosa, dorsal ectoderm, ventral (neurogenic) ectoderm and mesoderm (Rusch and Levine, 1996;

Stathopoulos and Levine, 2002). The boundaries of these domains are established by the activities of zygotic genes transcribed at different thresholds of the Dorsal gradient. One such zygotic gene is *decapentaplegic* (*dpp*), which is expressed in dorsal 40% of the cellular blastoderm (St Johnston and Gelbart, 1987). It encodes a secreted protein belonging to the TGFβ superfamily (Padgett et al., 1987). The *dpp* mutant embryo is strongly ventralized (Irish and Gelbart, 1987). Conversely, *dpp* overexpression expands the dorsal area (Ferguson and Anderson, 1992a; Ferguson and Anderson, 1992b). Thus, the Dpp protein acts as a dorsal morphogen in the *Drosophila* embryo. In cells that receive the Dpp signal, the cytoplasmic Mothers against dpp (Mad) protein is phosphorylated by activated Dpp receptors, and translocated to the nucleus, where phosphorylated Mad (pMad) regulates transcription of downstream genes (Raftery and Sutherland, 1999; Rushlow et al., 2001). Another zygotic gene, *short gastrulation* (*sog*), is expressed at lateral regions next to the *dpp*-expressing domain (François et al., 1994). This gene encodes an extracellular protein (François et al., 1994) that antagonizes the Dpp activity (Marqués et al., 1997; Ashe and Levine, 1999). The dorsal region is established by the relative activities of Dpp, Sog and other factors (Ashe et al., 2000).

Striking similarities in the mechanisms of DV patterning are known between *Drosophila* and vertebrates. Vertebrate homologs of *dpp* and *sog*, BMP2/4 and chordin, respectively, function to organize the DV pattern in a similar fashion although the ventral side of vertebrates corresponds to the dorsal side of *Drosophila* (Holley et al., 1995; De Robertis and Sasai, 1996; Ferguson, 1996; Holley and Ferguson, 1997). Combined with the distant phylogenetic relationship between *Drosophila* and vertebrates, it is generally thought that the origin of the DV axis is shared by most bilaterians. However, there are very few studies investigating how the embryonic DV axis is specified in bilaterian animals other than insects and vertebrates. Although homologs of *dpp*/BMP2/4 have been isolated in a wide range of metazoans, including the amphioxus, ascidian, sea urchin, gastropod, planarian and coral (Miya et al., 1997; Panopoulou et al., 1998; Orii et al., 1998; Angerer et al., 2000; Darras and Nishida, 2001; Hayward et al., 2002; Nederbragt et al., 2002), developmental analyses on these genes have not revealed plausible scenarios for the evolution of DV axis formation in the metazoans. It may be important to try to figure out the ancestral mode of DV patterning for the respective phyla or classes.

In non-insect arthropods, there is only limited knowledge concerning cellular and molecular mechanisms of early patterning. Molecular phylogenetics suggests that within the Arthropoda the Chelicerata are the living group phylogenetically most distant from the Insecta (Friedrich and Tautz, 1995; Hwang et al., 2001; Giribet et al., 2001; Cook et al., 2001). Comparative analysis is expected to provide data for understanding the ancestral developmental mechanisms of the arthropods. An increasing number of developmental genes have been isolated and examined in the spider and other chelicerates, illuminating similarities and differences between *Drosophila* and chelicerates (Telford and Thomas, 1998a; Telford and Thomas, 1998b; Damen and Tautz, 1998; Damen et al., 1998; Damen et al., 2000; Abzhanov et al., 1999; Stollewerk et al., 2001; Damen, 2002; Dearden et al., 2002).

In this study, in order to compare the mechanisms patterning the AP and DV axes of *Drosophila* and spider embryos, we used *Achaearanea tepidariorum*, which is easily accessible to analyzing the early embryogenesis. First, we describe a cluster of mesenchymal cells at the cumulus, which migrate from the center to the rim of the germ disc resulting in the transition from radial to axial symmetry. Next ,we isolated spider genes homologous to *Drosophila* early patterning genes, *dpp*, *fork head* (*fkh*), *orthodenticle* (*otd*; *oc* – FlyBase) and *caudal* (*cad*), and examined their expressions. In the *Drosophila* cellular blastoderm, *dpp* is expressed to specify the dorsal part (Irish and Gelbart, 1987; St Johnston and Gelbart, 1987; Ferguson and Anderson, 1992a; Ferguson and Anderson, 1992b), and *fkh*, *otd* and *cad* are expressed to specify the anterior and posterior terminal domains (Weigel et al., 1989; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Macdonald and Struhl, 1986; Wu and Lengyel, 1998). The expression patterns of the spider genes helped understand the early development of the embryo. We found that the mesenchymal cells at the cumulus expressed the *dpp* homolog. Furthermore, using the crossreacting antibody against phosphorylated Mad (pMad), we showed that the Dpp signal was received by germ disc epithelial cells. We suggest that at the cumulus, progressive mesenchymal-epithelial cell interactions involving the Dpp-Mad signaling cascade generate DV polarity in accordance with the AP axis formation. Our findings offer molecular support for the functional importance of the cumulus in the axial pattern formation of the spider embryo.

MATERIALS AND METHODS

Spider

The cosmopolitan house spider, *Achaearanea tepidariorum* (Araneae, Theridiidae), was used. Spiders were originally collected on the campus of Kyoto University (Kyoto, Japan). They were cultured in the laboratory over four generations in cycles of light (15 hours) and dark (9 hours) at 25°C, and fed with flies and crickets. Egg sacs were kept in 100 mm dishes with wet cotton. Hatched larvae were kept in the same dishes until they became fourth or fifth instars. They were then individually transferred to small glass tubes (15×70 mm). Several days after the final ecdysis, females were transferred to larger cups and mated with mature males. Within several days, the females made an egg sac containing approximately 200-300 eggs, and repeated the egg laying approximately once a week for two months. Living embryos were observed in halocarbon oil 700 (Sigma).

Scanning electron microscopy

Spider eggs were dechorionated with commercial bleach, followed by careful washing with distilled water. For fixation, the eggs were incubated for several hours or over night in a two-phase solution of heptane, 4% paraformaldehyde and 0.7% glutaraldehyde in C & G's balanced saline (55 mM NaCl, 40 mM KCl, 15 mM $MgSO_4$, 5 mM $CaCl_2$ and 10 mM Tricine, pH 6.9) at 4°C. The fixative was replaced by phosphate-buffered saline (PBS) with 0.1% Tween20 (PBS-Tween), and the vitelline membranes were removed with forceps and glass needles. Some of the samples, used to observe the inside of the egg, were cut by a razor blade, followed by a removal of the yolk. Then, the samples were post-fixed with 2.5% glutaraldehyde in PBS for 1 hour or longer. After several washes with PBS, they were dehydrated through an ethanol series, followed by a replacement with *t*-butylalcohol. They were then critical-point dried using a freeze-drying device, JFD-300 (JEOL), sputtered with an ion sputtering device, JFC-1500 (JEOL), and examined at 10 kV in a scanning electron microscope (SEM), JSM-5300LLV (JEOL). Photographs were taken with Polapan 572 (Polaroid).

Phalloidin staining

Eggs were fixed in the same fixative as used for the SEM preparation, or in a two-phase fixative of heptane and 4% paraformaldehyde in PBS for several hours or overnight at 4°C. After the removal of the vitelline envelopes, the eggs were stained with 1 U/ml phalloidin-fluorescein (Molecular Probes). The eggs fixed in the gulutaryaldehyde-containing fixative were structurally well preserved, but stained weakly as compared with those fixed in the other. Some of the samples were counterstained with 1 μM TOTO-3 (Molecular probes). The stained samples were observed under a Zeiss Axiophoto 2 microscope equipped with a BioRad laser confocal system (MRC1024).

cDNA cloning

To isolate spider homologs of the *Drosophila* genes, *dpp*, *fkh*, *otd* and *cad*, we initially performed degenerate PCR. Primers used for amplification of the genes were as follows:

the *dpp* forward primer, 5′ ga(t/c)gtnggntgg(a/g)a(t/c)ga(t/c)tgg 3′ (for amino acid sequence DVGW(N/D)DW);

the *dpp* reverse primer, 5′ cg(a/g)cancc(a/g)canccnacnac 3′ (for amino acid sequence VVGCGCR);

the *fkh* forward primer, 5′ ca(t/c)gcnaa(a/g)ccnccnta(t/c)(a/t)(g/c) 3′ (for amino acid sequence HAKPPYS);

the *fkh* reverse primer, 5′ gg(a/g)tgna(a/g)n(g/c)(a/t)cca(a/g)(t/a)a 3′ (for amino acid sequence (F/Y)W(T/S)LHP);

the *otd* forward primer, 5′ gnta(t/c)ccnga(t/c)at(t/c/a)tt(t/c)atg 3′ (for amino acid sequence RYPDIFM);

the *otd* reverse primer, 5′ gcnc(t/g)nc(t/g)(a/g)tt(t/c)tt(a/g)aacca 3′ (for amino acid sequence WFKNRRA);

the *cad* forward primer, 5′ gg(t/c)aa(a/g)acn(a/c)gnacnaa(a/g)ga 3′ (for amino acid sequence GKTRTKD);

and the *cad* reverse primer, 5′ tc(t/c)ttngcnc(t/g)nc(t/g)(a/g)tt(t/c)tg 3′ (for amino acid sequence QNRRAKE).

cDNA prepared from segmentation stage embryos was used as a template for the PCR amplifications. The cycles of PCR for *dpp* were: one cycle of 95°C for 5 minutes, 55°C for 2 minutes 30 seconds and 72°C for 40 seconds; and 35 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 40 seconds. For amplification of *fkh*, *otd* and *cad*, the annealing temperatures were changed to 45°C, 50°C and 50°C, respectively. Amplified fragments were subcloned using a TA-cloning® kit dual promoter (Invitrogen) and sequenced. Among them, candidates for the spider homologs of the *Drosophila* genes were found.

For cDNA library construction, two pools of polyA$^+$ RNA were prepared from three egg sacs containing embryos at different stages and just hatched prelarvae of *Achaearanea tepidariorum* using a QuickPrep™ Micro mRNA Purification Kit (Amersham Pharmacia Biotech). From each RNA pool, oligo-dT primed cDNA libraries were constructed using SuperScript™ Lamda System for cDNA Synthesis and Cloning (Gibco BRL) and Gigapack®III Gold Packaging Extract (Stratagene).

For library screening, digoxigenin (DIG)-labeled DNA probes for the PCR-amplified fragments were made using a PCR DIG Probe Synthesis Kit (Roche). The embryo and prelarvae cDNA libraries were screened with the probes in a high stringent condition to obtain full-length cDNAs for the candidate genes. Both strands of representative cDNA clones were sequenced and their open reading frames were determined. As shown in the text, the isolated genes were concluded to be *A. tepidariorum* orthologs of *Drosophila dpp*, *fkh*, *otd* and *cad*, which were designated *At.dpp*, *At.fkh*, *At.otd* and *At.cad*, respectively. The sequences are available from the DNA data bank of Japan (DDBJ) with the following Accession Numbers: *At.dpp*, AB096072; *At.fkh*, AB096073; *At.otd*, AB096074; *At.cad*, AB096075.

Phylogenetic analysis

The deduced amino acid sequences of the C-terminal region of the Dpp protein, the winged-helix region of Fkh and the homeodomains of Otd and Cad were manually aligned with the corresponding amino acid sequences of proteins from other species that were found using the BLAST search. Aligned sequences were used to construct phylogenetic trees by the neighbor-joining method (Saitou and Nei, 1987) using PHYLIP.

In situ hybridization and antibody staining

RNA probes for in situ hybridization were prepared using T7 RNA polymerase (Gibco BRL, or Stratagene) and DIG RNA Labeling Mix (Roche) according to the standard method. After dechorionation with bleach, embryos were fixed in a two-phase solution of heptane and 5.5% formaldehyde in PEMS (100 mM PIPES, 1 mM EDTA, 2 mM $MgSO_4$, pH 6.9). After fixation, the embryos were washed with PBS-Tween, followed by gradual replacement with methanol, and then the vitelline membranes were removed with forceps and glass needles. Alternatively, the vitelline membranes were removed in PBS-Tween before replacement with methanol. The former procedure protected the yolk mass from destruction, but the cumulus was hardly visible in the resultant samples. To observe the cumulus carefully, we followed the latter procedure, which allowed us to remove the yolk mass with the germ disc preserved. In the resultant samples, the cumulus was easily visible even after staining. Hybridization, washes and detection were performed in the same way as those for the *Drosophila* embryos (Lehmann and Tautz, 1994). For antibody staining, embryos were prepared in the same way as for the in situ hybridization staining. PS1 antibody raised against the phosphorylated human Smad1 C-terminal peptide (Persson et al., 1998) was used at a dilution of 1:500 to detect pMad protein. For the secondary antibody, biotin-conjugated anti-rabbit IgG (Amersham) was used at a dilution of 1:200. For detection, the elite ABC peroxidase kit (Vectastain) was used. For simultaneous detection for *At.dpp* RNA and the pMad protein, the in situ hybridization staining was followed by the antibody staining.

RESULTS

Early embryogenesis of *Achaearanea tepidariorum*

The following is a brief description of embryogenesis up to the early segmentaion stage of a house spider, *Achaearanea tepidariorum*, based on observations of the living embryos, a time-lapse movie of the embryogenesis (see Movie 1 at http://dev.biologists.org/supplemental/), and examinations performed in the past (Montgomery, 1909; Holm, 1940; Holm, 1952; Seitz, 1966). Eight stages were defined (Fig. 1). The spider egg is spherical in shape (Fig. 1). The morphological appearance at earliest stages do not predict the future embryonic axis. At stage 1, the nuclei divide deep from the egg surface to increase in number (Fig. 1A). Stage 2 begins with the arrival of the cleavage energids, which are composed of the nuclei and cytoplasmic components, at the periphery of the egg (Fig. 1B). During this stage, the energids continue to divide synchronously, being distributed evenly along the egg surface (Fig. 1B, C). At stage 3, the energids begin to shift towards one side of the egg along the surface (Fig. 1D). Within 10 hours, most of the energids become settled in half of the egg (Fig. 1E), entering stage 4. The energids give rise to a germ disc of relatively uniform epithelial cells. Although when the cellularization is completed in the species used has not been examined, a previous work on a closely related spider species, *Achaearanea japonica*, showed that every nucleus was already enclosed with plasma membrane at the 16-blastomere stage (Suzuki and Kondo, 1995). At the center of the germ disc, a white spot is seen (Fig. 1E). This is traditionally called the primary thickening (or cumulus anterior), at which

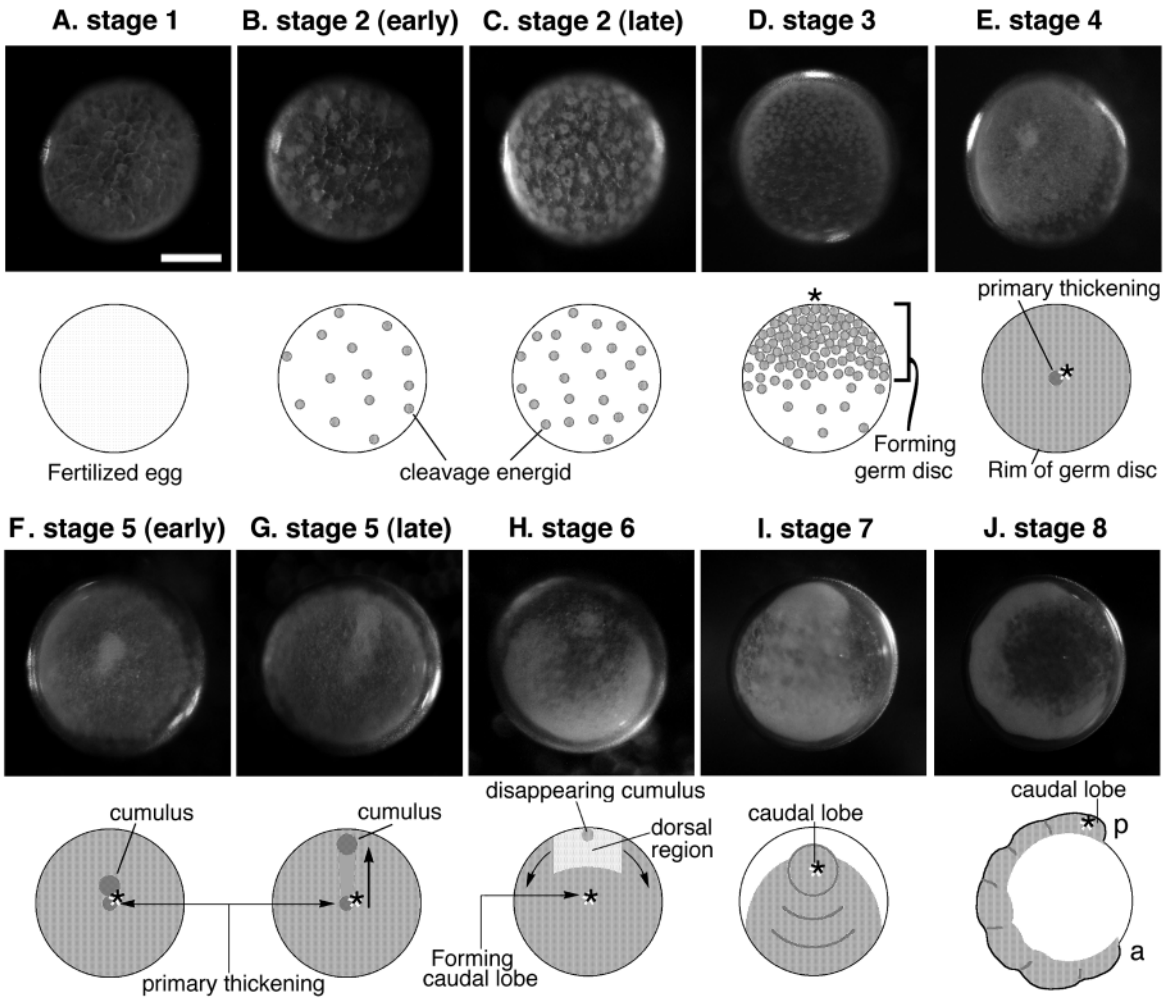

Fig. 1. Stages of early embryogenesis of the spider, *Achaearanea tepidariorum*. Photographs of living embryos at different stages are presented. These embryos were viewed from different angles to show morphologies characteristic of the given stages. Each embryo is schematically illustrated in the lower row. Asterisks indicate the corresponding site of the different stage embryos. White areas in the illustrations indicate yolk. Developmental schedule (at 25°C) is as follows: stage 1, 0-10 hours after egg laying (AEL); stage 2, 10-15 hours AEL; stage 3, 15-25 hours AEL; stage 4, 25-30 hours AEL; stage 5, 30-40 hours AEL; stage 6, 40-45 hours AEL; stage 7, 45-55 hours AEL; stage 8, 55-65 hours AEL. (A) Stage 1 embryo (about 5 hours AEL). Nuclear divisions occur deep in the egg. (B) Early stage 2 embryo (about 11 hours AEL). (C) Late stage 2 embryo (about 15 hours AEL). The energids reach the periphery of the egg, and undergo synchronous cleavages. (D) Stage 3 embryo (about 20 hours AEL). The energids shift toward one pole of the egg along the surface. (E) Stage 4 embryo (about 26 hours AEL). The energids settle and form a germ disc (large shaded circle). The primary thickening (small dark circle) is seen at the center of the germ disc. (F) Early stage 5 embryo (about 32 hours AEL). (G) Late stage 5 embryo (about 36 hours AEL). The cumulus appears from the center of the germ disc and moves straight to the rim (arrow). (H) Stage 6 embryo (about 42 hours AEL). The cumulus disappears, and the germ disc cells are rearranged migrating circumferentially (arrows). (I) Stage 7 embryo (about 50 hours AEL). The previous central region of the germ disc develops into the caudal lobe. Segmentation begins. Metameric morphologies are recognizable. The shape of the embryo is fan-like. (J) Stage 8 embryo (about 60 hours AEL). The germ band is formed, which elongates along the AP axis producing additional segments from the caudal lobe. a, anterior; p, posterior. Scale bar: 200 μm.

mesenchymal cells are located beneath the surface epithelium. We use the term primary thickening. A sign of the primary thickening is already visible at stage 3. At the primary thickening there is a slight indentation that is generally called the blastopore. We are not sure whether the mesenchymal cells arise from invagination through the blastopore. Stage 5 is the stage characterized by the cumulus. At the beginning of stage 5 the primary thickening becomes enlarged, and then a larger thickening appears from one side of the primary thickening (Fig. 1F). This larger thickening is the cumulus. The cumulus, which shows a bulge of the surface epithelium (Fig. 2A), shifts straight to the rim of the germ disc (Fig. 1G; see Movie 1 at http://dev.biologists.org/supplemental/). It takes about 6 hours for the cumulus to shift. However, the primary thickening becomes indistinct after the cumulus leaves the center of the germ disc (Fig. 1G). In about 75% of stage 4 embryos, the primary thickening was positioned almost at the center of the germ disc, whereas in the remaining 25%, it was positioned to one side of the center. In these cases, the direction of the cumulus movement was not correlated with the asymmetric position of the primary thickening. Stage 5 ends with the arrival of the cumulus at the rim of the germ disc. During stage 6,

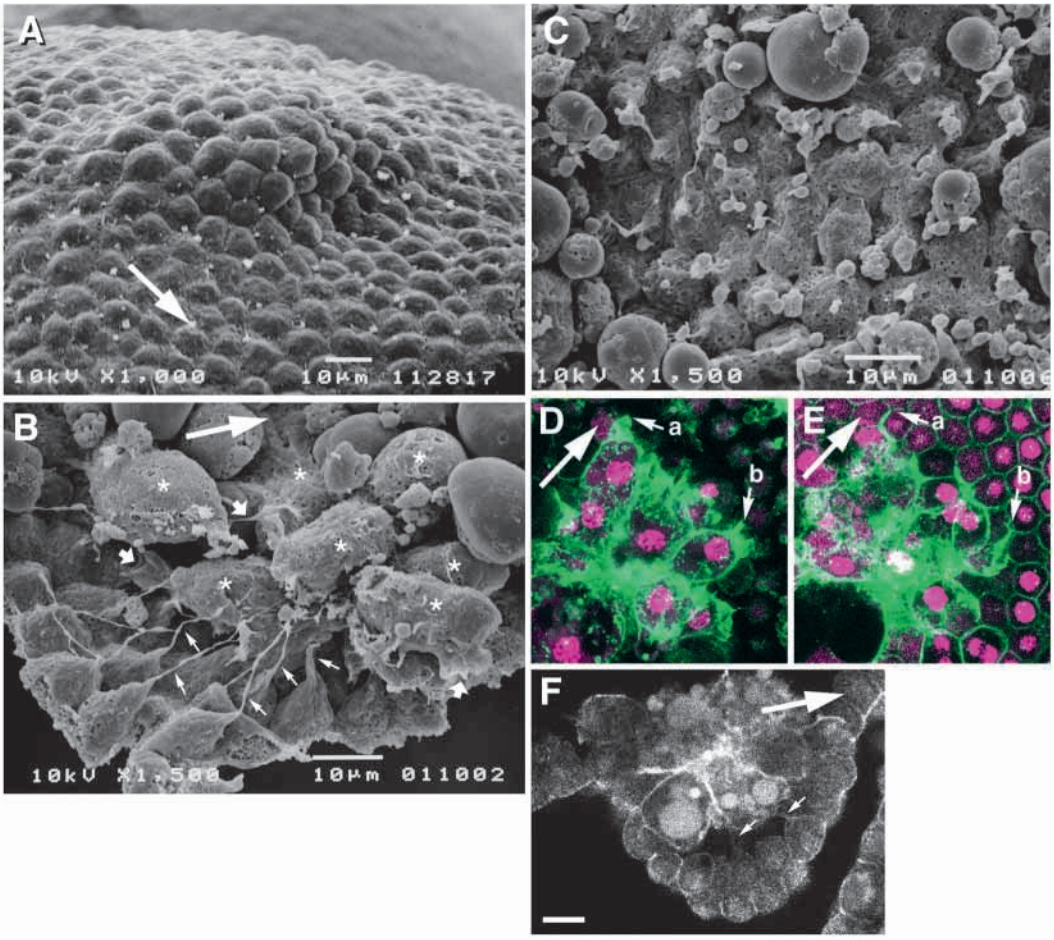

Fig. 2. Morphology of the cumulus. The direction of the cumulus movement is shown by the largest arrows (A,B,D-F). (A) SEM image showing the surface view of a cumulus. The epithelium is bulged at the cumulus. (B) SEM image showing the inside view of a cumulus. Asterisks indicate CM cells, which extend lamellipodia-like processes (thick arrows). Note that germ disc epithelial cells extend thin, long cytoplasmic projections like cytonemes (thin arrows) from their basal side onto the surface of the CM cells. A single epithelial cell has a single projection. (C) SEM image showing the inside view of germ disc epithelial cells far from the cumulus in the same embryo as in B. No cytoneme-like projections are seen. (D,E) Laser scanning microscopy (LSM) images of a cumulus stained with phalloidin (green) and TOTO-3 (purple). A series of optical sections separated by intervals of 0.52 µm were obtained to cover the epithelial and mesenchymal layers of the cumulus. D and E were constructed by overlaying 16 and nine successive optical sections, respectively, selected from the data set. D focuses on the CM cells, and E on the interface between the CM cells and the germ disc epithelial cells. Lamellipodia-like processes abundant with F-actin ingress into the spaces between the lateral surfaces of the epithelial cells as indicated by arrows (a and b). (F) LSM image showing a sagittal section of a cumulus stained with phalloidin-fluorescein. Cytoplasmic projections extend from the basal side of the epithelial cells are seen (arrows). Scale bar: 10 µm in A-C; 20 µm in D-F.

dynamic rearrangement of the surface epithelial cells takes place in the germ disc (Fig. 1H; see Movie 1 at http://dev.biologists.org/supplemental/) (Holm, 1940; Holm, 1952; Seitz, 1966). Peripheral cells migrate circumferentially toward the opposite side of the egg with respect to the disappearing cumulus (Fig. 1H), contributing to the development of the anterior region of the embryo. At stage 7, the germ disc is changed to a fan-like shape, and a metameric pattern begins to be morphologically recognized (Fig. 1I). The caudal lobe is prominent, which is derived from the cells around the center of the germ disc (see Movie 1 at http://dev.biologists.org/supplemental/) (Holm, 1940; Holm, 1952; Seitz, 1966). At stage 8, the germ disc is completely transformed to a germ band (Fig. 1J). Opisthosoma segments emerge one by one from the caudal lobe.

Morphological characterization of CM cells

Germ discs with the migrating cumulus were observed from the inside by SEM. Mesenchymal cells were found to be present at a position corresponding to the epithelial cell bulge of the cumulus (Fig. 2B, asterisks). We designated these cells cumulus mesenchymal (CM) cells. The CM cells were rather loosely associated with each other and showed rufflings of plasma membrane like lamellipodia (Fig. 2B, thick arrows). F-actin staining visualized the lamellipodia-like projections, some of which ingressed into the spaces between the columnar epithelial cells (Fig. 2D,E). Using the samples stained for F-actin and DNA, the number of the CM cells were counted. The average number was 8.7±1.1 (n=17 embryos). In addition to the CM cells, a small number of mesenchymal cells existed, associated with the basal surface of the epithelium but distributed differently from the CM cells (not shown).

Marked differences in morphologies were also observed among the epithelial cells. The epithelial cells located in the trail of the CM cells extended long, thin cytoplasmic projections from their basal side onto the surface of the CM cells (Fig. 2B, thin arrows). The epithelial cells positioned very close to the CM cells had short, thick projections. These cytoplasmic projections were faintly detectable by phalloidin-fluorescein (Fig. 2F). These observations remind one of the cytonemes described in *Drosophila* larval imaginal discs (Morata and Basler, 1999; Ramírez-Weber and Kornberg, 1999; Ramírez-Weber and Kornberg, 2000). In most other epithelial cells of the germ disc, no cytoneme-like projections were found (Fig. 2C).

A. Dpp/BMP2/4

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At.Dpp      RRHALYVDFSDVGWNDWIIAPPGYNAYFCHGDCPFPLPDHLNSTNHAIVQTLVNSANPAAVPRACCVPTELSPISMLYKDKFDNVVLKNYQDMVVEGCGCR
Dm.Dpp      RRHSLYVDFSDVGWDDWIVAPLGYDAYYCHGKCPFPLADHFNSTNHAVVQTLVNNMNPGKVPKACCVPTQLDSVAMLYLNDQSTVVLKNYQEMIVVGCGCR
Tc.Dpp      RRRQMYVDFGSVGWNDWIVAPLGYDAYYCGGECEYPIPDHMNSTNHAIVQSLVNSMKPKEVPGPCCVPTQLGQMSMLYLGSDGSVILKNYKEMVVVGCGCR
Sg.Dpp      RRHPLYVDFREVGWDDWIVAPPGYEAYYCHGDCPFPLSAHMNSTNHAVVQTLMNSVNPGLVPKACCVPTQLTSISMLYLDEESKVVLKNYHEMAVVGCGCR
Bf.BMP2/4  RRHSLYVDFSDVGWNDWIVAPPGYQAYYCHGECPFPLADHLNSTNHAIVQTLVNSVNPLAVPKACCVPTDLSPISMLYLNENDQVVLKNYQDMVVEGCGCR
Hr.BMPb     QRQDLYVDFSDVNWDDWIVAPIGYHAFYCNGECPFPLAEYMNATNHAIVQTLVNSVDPSLTPKPCCVPTELSPIAMLYVDECELVVLKTYQQMAVEGCGCR
Dr.BMP-2    RRHALYVDFSDVGWNEWIVAPPGYHAFYCHGECPFPLPDHLNSTNHAIVQTLVNSVNS-NIPKACCIPTELSPISLLYLDEYEKVILKNYQDMVVEGCGCR
Dr.BMP-4    RRHALYVDFSDVGWNDWIVAPPGYQAYYCHGECPFPLADHLNSTNHAIVQTLVNSVNT-NIPKACCVPTELSAISMLYLDETDRVVLKNYQEMVVEGCGCR
Dm.60A      QMQTLYIDFKDLGWHDWIIAPEGYGAFYCSGECNFPLNAHMNATNHAIVQTLVHLLEPKKVPKPCCAPTRLGALPVLYHLNDENVNLKKYRNMIVKSCGCH
Dm.Screw    ERLNFTVDFKELHMHNWVIAPKKFEAYFCGGGCNFPLGTKMNATNHAIVQTLMHLKQP-HLPKPCCVPTVLGAITILRYLNEDIIDLTKYQKAVAKECGCH
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B. Fkh/HNF-3

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At.Fkh      KFRRSLPHAKPPYSYISLITMAIQNSPQKMLTLNEIYQFIVDLFPFYRQNQQRWQNSIRHSLSFNDCFVKVARTPDKPGKGSFWALHPESGDMFENGCFLRRQKRFKCTKK
Dm.Fkh      TYRRSYTHAKPPYSYISLITMAIQNNPTRMLTLSEIYQFIMDLFPFYRQNQQRWQNSIRHSLSFNDCFVKIPRTPDKPGKGSFWTLHPDSGNMFENGCYLRRQKRFKDEKK
Tc.Fkh      TYRRSYTHAKPPYSYISLITMAIQNSPQKMLTLSEIYQFIMDLFPFYRQNQQRWQNSIRHSLSFNDCFVKVPRTPDKPGKGSFWSLHPDSGNMFENGCYLRRQKRFKDEKK
Bf.HNF3-1  AYRRSYTHAKPPYSYISLITMSIQSSPNKMVTLAEIYQFIMDLFPYYRQNQQRWQNSIRHSLSFNDCFVKVPRTPDRPGKGSYWTLHPEAGNMFENGCYLRRQKRFKCEKK
Hr.HNF-3    TYRRNYTHAKPPYSYISLITMAIQSSKQKMVTLSEIYQWIMDLFPFYRQNQQRWQNSIRHSLSFNDCFVKVARSPDKPGKGSYWALHQDAHNMFENGCYLRRQKRFKCKPK
Dr.Axial    TYRRSYTHAKPPYSYISLITMAIQQSPSKMLTLSEIYQWIMDLFPFYRQNQQRWQNSIRHSLSFNDCFLKVPRSPDKPGKGSFWTLHPDSGNMFENGCYLRRQKRFKCDKK
Dm.FD1      APHQNKEIVKPPYSYIALIAMAIQNAADKKVTLNGIYQYIMERFPYYRDNKQGWQNSIRHNLSLNECFVKVARDDKPGKGSYWTLDPDSYNMFINGSFLRRRRFKKKDV
Dm.FD3      SGSSGGPLVKPPYSYIALITMAILQSPHKKLTLSGICDFIMSRFPYYKDKFPAWQNSIRHNLSLNDCFIKVPREPGNPGKGNFWTLDPLAEDMFINGSFLRRRKRYKRAPT
Dm.FD4      PS ESYGEQKPPYSYISLTAMAIWSSPEKMLPLSDIYKFITDRFPYYRKNTQRWQNSLRHNLSFNDCFIKVPRRPDRPGKGAYWALHPQAFDMFENGSLLRRRKRFKLHKN
Dm.FD5      PLKMSYGDQKPPYSYISLTAMAIIHSPQRFVPLSEIYRFIMDQFPFYRKNTQKWQNSLRHNLSFNDCFIKVPRNVTKAGKGSYWTLHPMAFDMFENGSLLRRRKRFRVKQL
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C. Otd/Otx

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At.Otd      RKQRRERTTFTRAQLDILEALFQKTRYPDIFMREEVANQIRLAESRVQVWFKNRRAKCRQQ----LKFESL
Dm.Otd      RKQRRERTTFTRAQLDVLEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ----MNSDRI
Tc.Otd1     RKQRRERTTFTRAQLDLLEGLFAKTRYPDIFMREEVAVKINLPESRVQVWFKNRRAKCRQQ----EFANMA
Tc.Otd2     RKQRRERTTFTRAQLDVLEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ----SGWERK
Bf.Otx      RKQRRERTTFTRAQLDVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ----HKFQVL
Ci.Otx      RKQRRERTTFTRAQLDILEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ----WKFQVL
Dr.Otx2     RKQRRERTTFTRAQLDVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ----WKFQVL
Dm.Otp      NKQKRHRTRFTFAQLNELERCFSKTHYPDIFMREEIAMRIGLTESRVQVWFQNRRAKWKKR----NSNSVY
Dm.Prd      RKQRRCRTTFSASQLDELERAFERTQYPDIYTREELAQRTNLTEARIQVWFSNRRAKLRK ----FYPSWY
Dm.Gsb      RKQRRSRTTFSNDQIDALERIFARTQYPDVYTREELAQSTGLTEARVQVWFSNRRARLRK ----YPYFGF
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D. Cad/Cdx

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At.Cad      GRTRTKDKYRIVYTDHQRLELEKEFHY-SRYITIRRKVELAASLNLSERQIKIWFQNRRAKERRQAKKRGE
Dm.Cad      GKTRTKDKYRVVYTDFQRLELEKEYCT-SRYITIRRKSELAQTLSLSERQVKIWFQNRRAKERKQNKKGSD
Bm.Cad      GKTRTKDKYRVVYSDHQRLELEKEFHY-SRYITIRRKAELAVSLGLSERQVKIWFQNRRAKERKQVKKRGE
Tc.CadA     GKTRTKDKYRVVYTDHQRVELEKEFYY-SRYITIRRKAELANSLGLSERQVKIWFQNRRAKERKQVKKRGE
Tc.CadB     GKTRTKDKYRVVYTDLQRIELEKEFTFVSKYITIKRKSELAENLGLSERQIKIWFQNRRAKERKQNKKRIE
Hr.Cad      GKTRTKDKYRVVYSDHQRLELEKEFHF-SRYITIRRKSELAMQLSLSERQIKIWFQNRRAKERKISKKQTG
Dr.Cad1     GKTRTKEKYRVVYTDHQRLELEKEFHF-NRYITIRRKSELAVNLGLSERQVKIWFQNRRAKERKLIKKKLG
Dm.Dfd      QPGMEPKRQRTAYTRHQILELEKEFHY-NRYLTRRRRIEIAHTLVLSERQIKIWFQNRRVKWKKDNKLPNT
Dm.Scr      NANGETKRQRTSYTRYQTLELEKEFHF-NRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEHKMASM
Dm.Antp     GKCQERKRGRQTYTRYQTLELEKEFHF-NRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKENKTKGE
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Fig. 3. Characterization of amino acid sequences deduced from spider cDNA clones, *At.dpp*, *At.fkh*, *At.otd* and *At.cad*. Conserved amino acid residues in alignments are highlighted. Gaps introduced to optimize the alignments are indicated by dashes (A,D). (A) Amino acid sequence of the C-terminal domain of At.Dpp aligned with known Dpp/BMP2/4 family proteins and *Drosophila* 60A and Screw proteins. (Database Accession Numbers: Dm.Dpp, U63857; Tc.Dpp, U63132; Sg.Dpp, AF374725; Bf.BMP2/4, AF068750; Hr.BMPb, D85464; DrBMP-2, AF072456; DrBMP-4, D49972; Dm.60A, M84795; Dm.Screw, U17573.) (B) Amino acid sequence of the winged-helix (forkhead) domain of At.Fkh aligned with known Fkh/HNF-3 family proteins and *Drosophila* FD1 and FD3-FD5 proteins. (Database Accession Numbers: Dm.Fkh, J03177; Tc.Fkh, AF217810; Bf.HNF3-,1 X96519; Hr.HNF-3, AB007406; DrAxial, Z22762; Dm.FD1 and FD3-FD5, M96440 and M96442-M96444.) (C) Amino acid sequence of the homeodomain and C-terminal hexapeptide of At.Otd aligned with known Otd/Otx family proteins and *Drosophila* Otp, Prd and Gsb proteins. Dashes indicate the varied numbers of amino acid residues omitted. (Database Accession Numbers: Dm.Otd, X58983; Tc.Otd1 and 2, AJ223627 and AJ223614; Bf.Otx, AF043740; Ci.Otx, AF305499; DrOtx2, D26173; Dm.Otp, NM-079075; Dm.Prd, M14548; Dm.Gsb, NM-079139.) (D) Amino acid sequence of the homeodomain of At.Cad aligned with known Cad/Cdx family proteins and *Drosophila* Dfd, Scr and Antp proteins. (Database Accession Numbers: Dm.Cad, NM-134301; Bm.Cad, D16683; Tc.CadA and B AJ00542 and AJ005422; DrCad1, X66958, Dm.Dfd, X05136; Dm.Scr, X14475; Dm.Antp, M20704.) Abbreviated species are as follows: Dm, *Drosophila melanogaster*; Tc, *Tribolium castaneum*; Sg, *Schistocerca gregaria*; Bm, *Bombyx mori*; Bf, *Branchiostoma floridae*; Hr, *Halocynthia roretzi*; Ci, *Ciona intestinalis*; Dr, *Danio rerio*.

Cloning of spider homologs of *Drosophila dpp*, *fkh*, *otd* and *cad*

To search for developmental genes expressed in early spider embryos, we isolated cDNA clones for *A. tepidariorum* homologs of *Drosophila dpp*, *fkh*, *otd* and *cad*. Details for each cDNA clone are described below and in the Materials and Methods. Phylogenetic trees were constructed from the alignments shown in Fig. 3 (see Fig. S1 at http://dev.biologists.org/supplemental/).

At.dpp

The isolated cDNA clone encodes a polypeptide of 370 amino acids with a putative signal sequence and a putative proteolytic cleavage site (Panganiban et al., 1990) between 255 and 256 amino acid residues. The C-terminal 102 amino acid residues of the deduced protein shows 65% and 74% identity with the corresponding region of *Drosophila* Dpp and zebrafish BMP2, respectively, and lower percentages of identity with those of *Drosophila* 60A (50% identity) and Screw (42% identity) (Fig.

3A). The topology of a phylogenetic tree constructed using amino acid sequences from the C-terminal regions of the TGFβ superfamily proteins strongly suggested that the isolated gene was an ortholog of *Drosophila dpp*. It was designated as *At.dpp*.

At.fkh

Drosophila fkh is a winged-helix domain (forkhead domain) containing transcription factor expressed in several embryonic tissues such as hindgut, stomodeum and yolk (Weigel et al., 1989). The cDNA clone isolated from the spider predicts a protein of 406 amino acids which contains a winged-helix domain 82% identical to that of *Drosophila* Fkh and 83% to that of zebrafish Axial. The protein is more closely related to *Drosophila* Fkh than to other *Drosophila* winged-helix domain-containing proteins (Fig. 3B). In addition to the winged-helix domain, two other short domains at the C termini, which were conserved in most of the known Fkh/HNF3 class proteins (Lai et al., 1991), were found in the spider protein (not shown). Phylogenetic analysis based on the winged-helix domains confirmed that the isolated gene was an ortholog of *Drosophila fkh*. It was designate as *At.fkh*.

At.otd

Drosophila otd encodes a Paired-type homeodomain protein, which is expressed in the head region (Finkelstein et al., 1990; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). The cDNA clone isolated from the spider encodes a protein of 303 amino acids that contains a homeodomain. This homeodomain closely resembles those of *Drosophila* and other animal Otd/Otx proteins, but less closely resembles those of *Drosophila* Orthopedia (Otp), Paired (Prd) and Gooseberry (Gsb) (Fig. 3C). The C-terminal sequence of six amino acids, LKFESL, has an affinity to the sequence, W(K/R)FQVL, which is found at the C termini of most of the known Otd/Otx proteins except insect Otd proteins. Phylogenetic analysis based on the homeodomains confirmed that the isolated gene was an ortholog of *Drosophila otd*. It was designated as *At.otd*.

At.cad

Drosophila cad encodes a homeodomain protein that is expressed from the earliest stage of embryogenesis and is required for normal posterior development (Mlodzik et al., 1985; Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987; Wu and Lengyel, 1998). The cDNA isolated from the spider encodes a protein of 300 amino acids that contains a homeodomain. This homeodomain is highly similar to those of *Drosophila* and other animal Cad/Cdx proteins, but less similar to those of *Drosophila* Deformed (Dfd), Sex comb reduced (Scr) and Antennapedia (Antp) (Fig. 3D). Phylogenetic analysis based on the homeodomains confirmed that the isolated gene was an ortholog of *Drosophila cad*. It was designated as *At.cad*.

Expression of *dpp* in the CM cells

We examined the expression patterns of the isolated genes in stage 4-7 embryos by whole-mount in situ hybridization. Transcripts for *At.dpp* were not detected in stage 4 embryos, but were detected in stage 5 embryos. The signal was seen as a spot in the germ disc. The spot of *At.dpp* expression, which was found at different positions from embryo to embryo (Fig. 4A-D), corresponded to the position of the cumulus. The variation is probably due to variation in the age of the embryos. Even in embryos with the cumulus positioned almost at the center of the germ disc, *At.dpp* transcripts were already detectable (Fig. 4A,B), although we are not sure whether *the At.dpp* signal was asymmetric or not with respect to the position of the blastopore. Magnified images showed that *At.dpp* transcripts were expressed in the CM cells, but not in the surface epithelial cells (Fig. 4E,F). The number of *At.dpp*-positive cells was 9.3±2.0 (n=16), which was comparable with

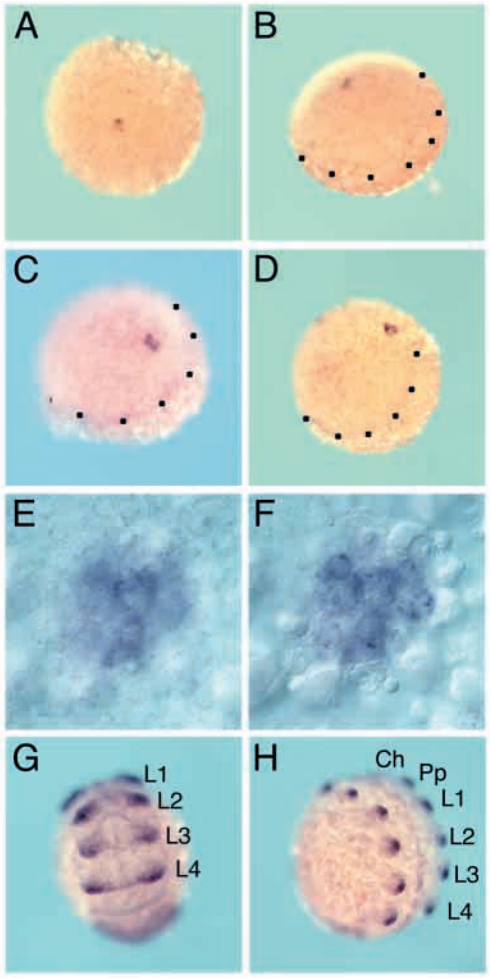

Fig. 4. Expression of *At.dpp* transcripts in stage 5 embryos revealed by whole-mount in situ hybridization. (A,B) Early stage 5 embryo viewed from the top (A) and the lateral side (B) of the germ disc. The signal is seen as a spot at the center of the germ disc. The rim of the germ disc is marked by dots in B as well as in C and D. (C) Mid stage 5 embryo. A spot of signal is seen at an intermediate position between the center and the rim of the germ disc. (D) Late stage 5 embryo. A spot of signal is seen close to the rim of the germ disc. The spots of *dpp* expression correspond to the positions of the cumulus in the embryos. (E,F) Close-up of the cumulus in a stage 5 embryo. E focuses on the surface epithelial cell layer and F on the CM cells. The signal is seen in the CM cells (F) but not the surface epithelial cells (E). (G,H) Embryos at about 65 hours (G) and 75 hours (H) AEL. The strong signal of *At.dpp* is seen in developing limb buds (G) and the tip of the extending limbs (H). The ventral midline is weakly stained (H). Ch, chelicerae; Pp, pedipalp; L1-4, leg segments 1-4.

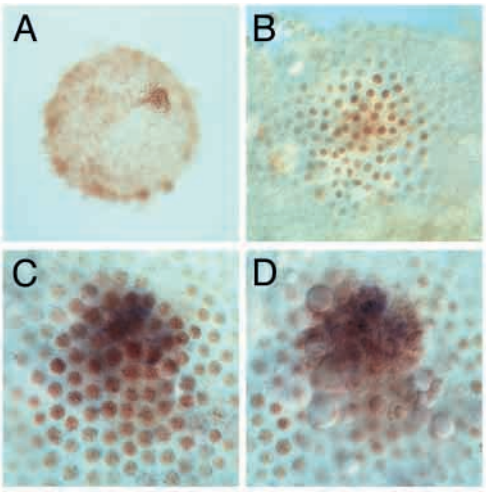

Fig. 5. Expression of pMad in stage 5 embryos. Embryos were stained with the PS1 antibody. (A) Mid stage 5 embryo viewed from the top of the germ disc. (B) Close-up of the same embryo as in A, flat-mounted. Nuclei of germ disc epithelial cells, but not CM cells, at and around the cumulus are stained. Note that the levels of signal are graded in a concentric manner. (C,D) Mid stage 5 embryo double-labeled for pMad (brown) and *At.dpp* transcripts (purple). C focuses on the surface epithelial cell layer and D on the CM cells. Note that the highest levels of pMad are observed just above the CM cells, expressing *At.dpp* transcripts.

the number of CM cells counted in the embryos stained for F-actin and DNA (see above). *At.dpp* transcripts were probably expressed in all the CM cells. At late stage 6, when the cumulus disappeared, the spot of *At.dpp* expression was not detected (not shown). At later stages, *At.dpp* began to be expressed in developing limb buds (Fig. 4G), and the expression persisted at the extending limbs (Fig. 4H). This *At.dpp* expression resembles that of *dpp* homologs in insect limbs (Sanchez-Salazar et al., 1996; Niwa et al., 2000; Dearden and Akam, 2001), suggesting a conserved function of *dpp* in limb bud formation between the insects and spiders.

Localized phosphorylation of Mad in the epithelium

To identify the cells potentially responding to the *At.dpp*-expressing CM cells in the spider embryo, we used the PS1 antibody, which was raised against the phosphorylated human Smad1 C-terminal peptide (Persson et al., 1998) and crossreacts with *Drosophila* pMad (Tanimoto et al., 2000). Staining with this antibody visualizes nuclei of the cells responding to the Dpp signal in *Drosophila* (Tanimoto et al., 2000; Rushlow et al., 2001; Dorfman and Shilo, 2001). In the staining of stage 5 spider embryos with PS1, specific signals were obtained in the germ disc epithelium. Only the epithelial cells located at and around the cumulus, but not the CM cells, showed stained nuclei (Fig. 5A,B). Accompanied by the cumulus, the pMad-positive region shifted in the germ disc epithelium. These indicated that the expression of nuclear pMad was transient in the epithelial cells. Double labeling for pMad and *At.dpp* transcripts confirmed that the region of Mad phosphorylation in the epithelium overlay the *At.dpp*-expressing CM cells (Fig. 5C,D). There was a concentric gradient of Mad phosphorylation within the positive region, peaking at the epithelial cells closest to the CM cells (Fig. 5B-D).

Expression of *fkh* in three different populations of cells

Expression of *At.fkh* was detected in three populations of cells. The first population was mesenchymal cells dispersed around the center of the germ disc at stage 5 (Fig. 6B-D). These cells probably derived from the *At.fkh*-positive cells at the primary thickening at stage 4 (Fig. 6A). Judging from their distribution patterns, these mesenchymal cells were different from the CM cells. The second population was cells at the rim of the germ disc (Fig. 6B,C,E,G). These cells appeared to migrate circumferentially and settle to the anterior region (Fig. 6H, part a) in a similar way to the *At.otd*-expressing cells (see below). The third population was surface epithelial cells at and around the cumulus approaching the rim of the germ disc at late stage 5 (Fig. 6E,F). During stage 6 and 7, the *At.fkh*-expressing cells spread over the yolk on the dorsal surface

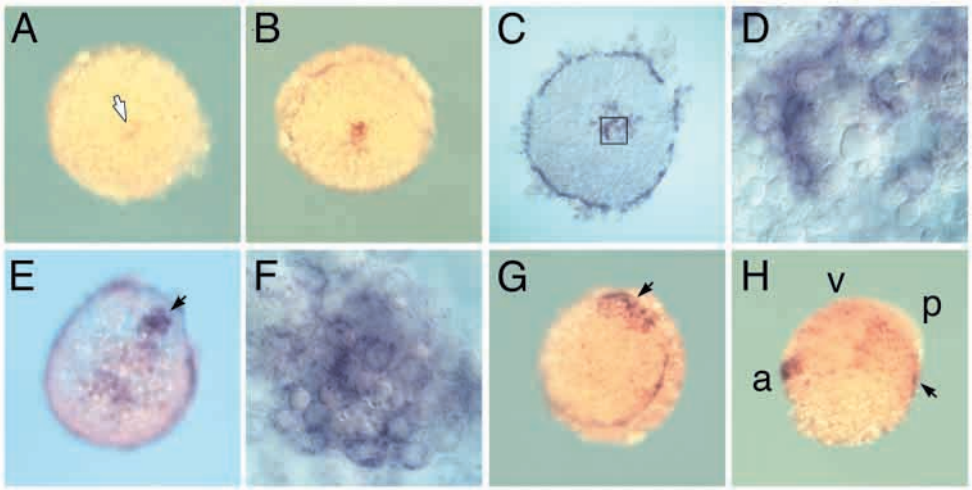

Fig. 6. Expression of *At.fkh* transcripts revealed by whole-mount in situ hybridization. (A) Stage 4 embryo viewed from the top of the germ disc. *fkh* transcripts are found around the blastopore (white arrow). (B) Early stage 5 embryo. Expression of *fkh* transcripts are seen in cells dispersing from the center of the germ disc and in cells encircling the germ disc. (C,D) Mid-stage 5 embryo flat mounted. (D) High magnification of the boxed area in C, focused on the mesenchymal cell layer. The *At.fkh*-positive cells are mesenchymal cells located below the surface epithelial cell layer. (E,F) Late stage 5 embryo. The cumulus is magnified in F. In addition to the dispersing mesenchymal cells and the peripheral cells, expression of *fkh* transcripts is observed in epithelial cells at the cumulus (arrow in E). (G,H) Stage 6 (G) and 7 (H) embryos. Expression of *fkh* transcripts is observed in the emerging dorsal region of the embryos (arrows), where the *At.fkh*-positive cells appear to spread over the yolk. The peripheral *At.fkh*-positive cells appear to migrate circumferentially to the emerging anterior region of the embryo (H, a). a, anterior; p, posterior; v, ventral.

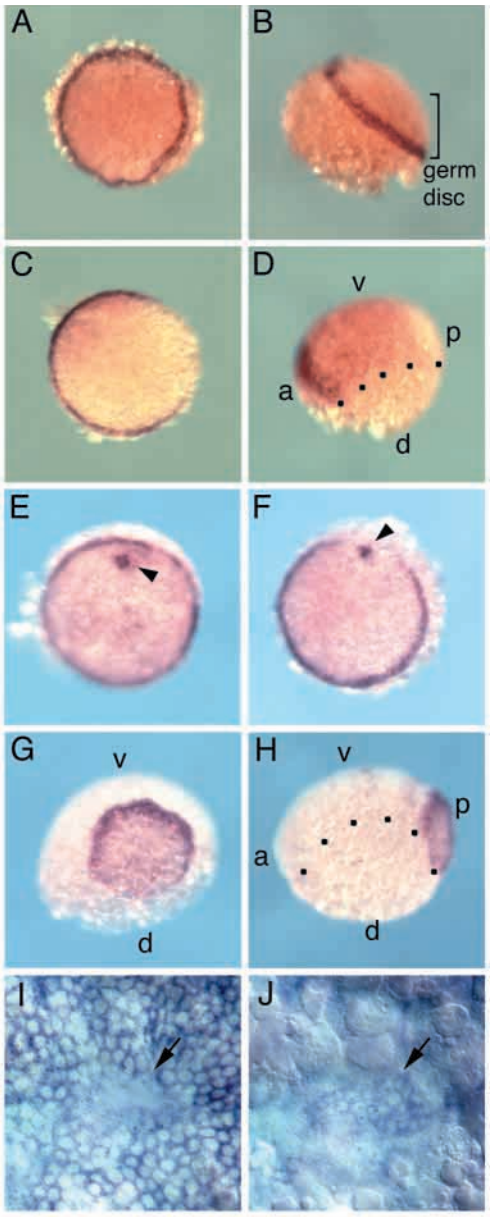

Fig. 7. Expression of *At.otd* and *At.cad* transcripts. Embryos stained by whole-mount in situ hybridization with the *At.otd* probe (A-D), *At.otd* and *At.dpp* mixed probes (E,F) or *At.cad* probe (G-J). (A,B) Stage 5 embryo viewed from the top (A) and the lateral side (B) of the germ disc. Expression of *At.otd* transcripts is observed in peripheral cells of the germ disc epithelium, three or four cells wide. (C) Stage 6 embryo viewed from the top of the germ disc. The pattern of *At.otd* expression is changed to an 'open' circle. (D) Stage 7 embryo viewed laterally. Expression of *At.otd* is seen at the emerging anterior region of the embryo. (E,F) Late stage 5 (E) and early stage 6 (F) embryos. The pattern of the *At.otd*-positive cells becomes the 'open' circle at almost the same time when the CM cells expressing *At.dpp* (arrowheads) reach the rim of the germ disc. (G,H) Stage 7 embryos viewed posteriorly (G) and laterally (H). Expression of *At.cad* transcripts is found at the most posterior region of the embryo corresponding to the caudal lobe. (I,J) High magnifications of the posterior end of a stage 7 embryo flat-mounted. I focuses on the surface of the embryo, and J on the inside. A pit is found at the center of the *At.cad*-expressing region (arrow in I). Below the pit, there is a population of cells expressing *At.cad* transcripts (arrow in J). Mesenchymal cells not expressing *At.cad* transcripts are also found. The margin of the forming germ band is indicated by dots in D and H. a, anterior; p, posterior; d, dorsal; v, ventral.

of the embryo (Fig. 6G,H, arrow; see Movie 1 at http://dev.biologists.org/supplemental/).

Expression of *otd* in the future anterior region

In stage 5 embryos, transcripts for *At.otd* were expressed in the peripheral cells of the germ disc epithelium, three or four cells wide in a circle (Fig. 7A,B). At stage 6, when the germ disc began to be rearranged, *At.otd* expression was observed in an 'open' circle (Fig. 7C). This change in the pattern of *At.otd* expression appeared to be associated with the cumulus approaching (Fig. 7E,F), which might negatively regulate the expression and/or induce the circumferential migration of the *At.otd*-positive cells. By the end of stage 6, the *At.otd*-expressing cells settled in the future head region (Fig. 7D). *At.otd*-negative cells, which possibly included the *At.fkh*-positive cells described above (Fig. 6H), were present more anteriorly than the *At.otd*-positive cells.

Expression of *cad* in the caudal lobe

No significant signal of *At.cad* expression was observed in stage 4-6 embryos. In stage 7 embryos, *At.cad* transcripts were found in the caudal lobe (Fig. 7G,H). At the center of the *At.cad*-expressing domain, a pit was present (Fig. 7I). *At.cad*-positive cells were also present underneath the pit (Fig. 7J), implying an invagination through the pit.

DISCUSSION

Mesenchymal-epithelial cell interactions at the cumulus

In this work we have described the early embryogenesis of the spider with special attention to the cumulus. At the beginning of stage 5, the cumulus arose from the primary thickening (Fig. 1F). The cumulus was observed as a cellular thickening that consisted of clustered mesenchymal cells (CM cells) and germ disc epithelial cells (Fig. 2A,B). SEM and fluorescent microscopy visualized the physical interactions between the CM cells and the germ disc epithelial cells (Fig. 2), which may have contributed to the bulge formation. The CM cells migrated on the basal surface of the epithelium, whereas the germ disc epithelial cells appeared not to change their positions. Taken together, the movement of the cumulus indicates the migration of the clustered CM cells accompanied with the bulge formation of germ disc epithelial cells.

We showed that the CM cells, but not germ disc epithelial cells, expressed *At.dpp* transcripts (Fig. 4E,F). Two lines of evidence suggest that the germ disc cells receive the Dpp signals from the CM cells. First, the nuclei of germ disc cells near the CM cells represented graded levels of pMad (Fig. 5). In *Drosophila* and vertebrates, phosphorylation and nuclear translocation of Mad/Smad proteins are shown to be caused by the activated specific receptors for Dpp/BMP ligands (Raftery and Sutherland, 1999). During migration, the CM cells were always centered in the pMad-positive circular region (Fig. 5),

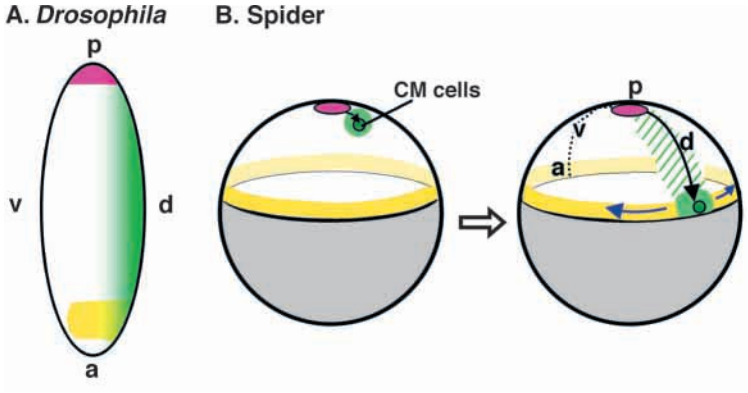

Fig. 8. Illustrations showing different modes of axial patterning of the *Drosophila* and spider embryo. The *Drosophila* cellular blastoderm stage (A) and the spider germ disc stage (B) are compared. a, anterior; p, posterior; v, ventral; d, dorsal. In each illustration, the *otd*- or *At.otd*-expressing anterior region of the embryo is indicated by yellow, and the posterior end of the embryo by purple. The area shown in gray indicate yolk (B). (A) In the *Drosophila* embryo, *dpp* is expressed in blastoderm cells at the 40% most dorsal region (green). This expression initiates simultaneously along the AP axis. In the same cell population that *dpp* is expressed, phosphorylation of Mad is induced. (B) In the spider embryo, future anterior cells (yellow) are located at the rim, whereas future posterior cells (purple) that form the caudal lobe exist around the center of the germ disc. The CM cells, which express *At.dpp* (green), migrate from the center to the rim of the germ disc (black arrow), progressively inducing phosphorylation of Mad in epithelial cells (light green). The area that once expressed pMad is shaded in green (right panel). It develops into the dorsal tissues. The site diametrically opposite to the cumulus is defined as the anterior pole (a, right panel). Broken line indicates the ventral midline; blue arrows indicate the directions of cell movement during stage 6.

implying that the appearance of pMad in the germ disc epithelium depended on the CM cells. Second, the germ disc epithelial cells directly contacted the CM cells by using cytoneme-like projections, which were extended from the basal side of the epithelial cells onto the surface of the CM cells (Fig. 2B). In *Drosophila* larval imaginal discs, the cytonemes, which are projected from cells receiving signals, are thought to be the place for cell-cell communication (Morata and Basler, 1999; Ramírez-Weber and Kornberg, 1999; Ramírez-Weber and Kornberg, 2000). Similar to this case, the germ disc epithelial cells were likely to use cytoneme-like projections to receive the Dpp signals from the CM cells. Taken together, we suggest that the cumulus is a place for mesechymal-epithelial cell interactions that are involved in the pattern formation of the spider embryo.

Nuclear pMad/Smad drives transcription of specific downstream genes (Raftery and Sutherland, 1999; Rushlow et al., 2001). The expression of *At.fkh* in an area of the germ disc associated with the migrating CM cells (Fig. 6E,F) appeared to overlap that of pMad (Fig. 5). This suggested that *At.fkh* is a potential downstream target gene for the Dpp-Mad signaling cascade. The *At.fkh*-expressing cells at the cumulus, spreading over the yolk during stage 6 and 7 (Fig. 6G,H), appeared to contribute to extra-embryonic tissue.

Similarities and differences in body axis formation of the *Drosophila* and spider embryos

Expression patterns of spider homologs of *Drosophila* region-specific genes, shown in this study, offer molecular clues for determining homologous domains in the early spider and *Drosophila* embryos (Fig. 8). In the *Drosophila* cellular blastoderm, *otd* is expressed at a region close to the anterior end (Finkelstein and Perrimon, 1990), whereas *At.otd* was expressed at a peripheral region of the germ disc encircled along the equator of the spider egg (Fig. 7A,B). Of these *At.otd*-expressing cells, cells around the cumulus might lack the *At.otd* expression (see above), but the remaining *At.otd*-expressing cells likely migrated circumferentially during stage 6 (Fig. 7C,F; see Movie 1 at http://dev.biologists.org/supplemental/) and settled to the anterior region of the germ band (Fig. 7D). *Drosophila cad* is expressed at a region close to the posterior end in the cellular blastoderm (Macdonald and Struhl, 1986), whereas *At.cad* was expressed in the caudal lobe (Fig. 7G,H), which was derived from the central area of the germ disc (see Movie 1 at http://dev.biologists.org/supplemental/). These results, together with previous observations of spider embryos (Holm, 1940; Holm, 1952; Seitz, 1966), strongly suggest that the peripheral region of the germ disc corresponds to the anterior end of the *Drosophila* embryo, and the central region corresponds to the posterior end. It is possible that in the spider germ disc, the AP positional information pre-exists as a series of concentric circles. Based on this topology, *At.fkh*-expressing cells were located at the future anterior and posterior ends of the stage 5 embryo (Fig. 6B,C), similar to the pattern of *fkh* expression in the *Drosophila* cellular blastoderm (Weigel et al., 1989) and in the early embryo of other insects, *Bombyx* and *Tribolium* (Kokubo et al., 1996; Schröder et al., 2000). As the *fkh*-expressing cells become foregut and hindgut in these insects (Weigel et al., 1989; Kokubo et al., 1996; Schröder et al., 2000), the two populations of *At.fkh*-expressing cells are probably fated to be gut precursors.

One possibly important difference in the mode of AP axis formation is that in the spider embryo, it appears to occur in accordance with DV axis formation, whereas in the *Drosophila* embryo, the AP and DV axes are formed independently. Although a concentric series of AP positional information may pre-exist on the spider germ disc, the expression patterns of the AP patterning genes do not allow one to find the AP axis in the early germ disc. The anterior pole of the spider embryo can be defined only after the onset of the cumulus movement (Fig. 8).

Drosophila dpp and probably other insect *dpp* homologs are involved in DV patterning of the embryo. *dpp* is expressed in the dorsal ectoderm during the germband extending stages, and this expression probably contributes to pattern formation within segments, such as positioning of the limb buds (Sanchez-Salazar et al., 1996; Niwa et al., 2000; Dearden and Akam, 2001). This role of *dpp* is probably conserved between the insects and spiders, as suggested by the later expression of *At.dpp* (Fig. 4G,H). At least in *Drosophila*, however, the earliest function of *dpp* is the specification of the DV pattern in the cellular blastoderm, not within segments of the germband (Ferguson and Anderson, 1992a; Rusch and Levine, 1996). The most dorsal region of the cellular blastoderm,

where *dpp* is expressed at the strongest level, becomes extra-embryonic tissue (the amnioserosa), and the next regions become dorsal ectoderm in *Drosophila*. In the spider, the area of the germ disc that the *At.dpp*-expressing CM cells have directly influenced during their migration (Figs 1, 2, 5) develops into dorsal structures including the extra-embryonic tissue. These may suggest a conserved function of *dpp* for dorsal fate specification of the early embryo. The expression of *At.dpp* in the CM cells (Fig. 4) is probably comparable with the dorsal expression of *dpp* in the *Drosophila* cellular blastoderm, and might be related to the expression of *dpp* in an early cell population fated to be extra-embryonic tissue in more basal insect embryos (Sanchez-Salazar et al., 1996; Dearden and Akam, 2001).

In the *Drosophila* blastoderm the expression of *dpp*, as well as some other zygotic genes involving DV patterning, is initiated in an asymmetric manner according to the gradient of the nuclear Dorsal protein peaking at the most ventral region (Rusch and Levine, 1996; Stathopoulos and Levine, 2002). In the spider embryo, however, the early detectable asymmetry was in the migration of the *At.dpp*-expressing CM cells rather than the expression of *At.dpp* itself. What mechanisms regulate the CM cell migration? The answer to this question may be the key to the developmental origin of the DV axis of the spider. A localized cue(s) that attracts or repulses the CM cells might pre-exist on the germ disc. Alternatively, the CM cells might sense only the AP positional information. In the latter case, the direction of the cell migration is determined at the start point randomly, or according to a local unevenness. Further molecular investigations are needed to find the earliest asymmetries potentially present in the germ disc and primary thickening.

Two important differences concern the Dpp signal between *Drosophila* and the spider (Fig. 8). First, the Dpp signal is produced and transduced within the surface epithelial cells in the *Drosophila* embryo (Dorfman and Shilo, 2001), in contrast to the spider embryo, in which the Dpp signal is produced by the mesenchymal cells (the CM cells) (Fig. 4), and is transmitted to the surface epithelial cells (Fig. 5). Second, the activation of the Dpp-Mad signaling pathway takes place simultaneously along the AP axis in the *Drosophila* embryo (Dorfman and Shilo, 2001), but took place progressively from the center to the rim of the germ disc in the spider embryo (Figs 4, 5). These differences have implications for evolutionary change in the mechanism governing DV axis formation.

dpp/BMP2/4 class genes have been identified in non-chelicerate animals, including insects and non-arthropod invertebrates, and their expression patterns have been examined in early embryos (Sanchez-Salazar et al., 1996; Miya et al., 1997; Panopoulou et al., 1998; Angerer et al., 2000; Niwa et al., 2000; Darras and Nishida, 2001; Dearden and Akam, 2001; Hayward et al., 2002). However, of the cells expressing the *dpp*/BMP2/4 homologs in early embryos, none resembles the spider CM cells in morphology and behavior. Within the Chelicerata, the horseshoe crab (Sekiguchi, 1973), as well as many spider species (Montgomery, 1909; Holm, 1940; Holm, 1952; Seitz, 1966), shows a cellular thickening similar to the cumulus in early embryogenesis. Even in early embryos of myriapod species, a cumulus-like cell mass has been reported (Heymons, 1901; Sakuma and Machida, 2002). In these cellular structures, cells that correspond to the CM cells described in this study might be present. Studies on *dpp* homologs will help to determine whether they are homologous to the spider cumulus. To understand the ancestral mode of DV patterning for the arthropods, the evolutionary and developmental origin of the CM cells is an important subject to be studied. For this purpose, crustaceans, which are suggested to be a sister group of insects (Friedrich and Tautz, 1995; Hwang et al., 2001; Giribet et al., 2001; Cook et al., 2001), are not negligible. Early patterning of crustacean embryos at the cellular level is now being studied (Wolff and Scholtz, 2002; Gerberding et al., 2002).

Regulative development of the early spider embryo

If it is assumed that common cellular and molecular mechanisms govern the early development of the spiders, *Agelena labyrinthica* and *Achaearanea tepidariorum*, the organizing activity of the cumulus demonstrated by Holm (Holm, 1952) might be explained on the basis of our findings. He removed the cumulus resulting in ventralized embryos with the AP pattern retained to some extent. Loss of the source of Dpp signals might account for the ventralization. Transplanting a part of the cumulus ectopically resulted in twin embryos. Two moving sources of Dpp signals might set up two separate fields defined by positional values in the germ disc. Combining our findings and Holm's experimental data, it is strongly suggested that the cumulus plays a central role in the axial pattern formation of the spider embryo. Our molecular evidence clearly indicates that the spider cumulus is not homologous to the organizing center of vertebrate embryos, in which the activity of Sog/chordin, the Dpp/BMP2/4 antagonist, is dominant (Sasai et al., 1994). Studies on spider homologs of Sog/chordin are needed for a better understanding of DV patterning in the spider embryo.

The experiments by Holm (Holm, 1952) and experiments on other spiders (Sekiguchi, 1957; Seitz, 1966; Seitz, 1970), indicate that the spiders adopt more regulative development than *Drosophila*. Like the spiders, the development of the horseshoe crab (Itow et al., 1991) and some insects (Sander, 1976) seems to be regulative. Probably in these arthropod species, the axial pattern formation largely relies on cell-cell interactions. In this study, we showed Dpp-mediated interactions between mesenchymal and epithelial cells at the cumulus. Probably, subsequent cell-cell interactions specify the fates of individual cells in the germ disc. Further investigations on patterning genes in the spider embryo will clarify the molecular basis for the regulative development of chelicerate embryos, which may give hints about the ancestral mode of arthropod development.

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