Axis specification in the spider embryo: dpp is required for radial-to-axial symmetry transformation and sog for ventral patterning

Yasuko Akiyama-Oda1,2,* and Hiroki Oda1,*

The mechanism by which Decapentaplegic (Dpp) and its antagonist Short gastrulation (Sog) specify the dorsoventral pattern in Drosophila embryos has been proposed to have a common origin with the mechanism that organizes the body axis in the vertebrate embryo. However, Drosophila Sog makes only minor contributions to the development of ventral structures that hypothetically correspond to the vertebrate dorsal somite where the axial notochord forms. In this study, we isolated a homologue of the Drosophila sog gene in the spider Achaearanea tepidariorum, and characterized its expression and function. Expression of sog mRNA initially appeared in a radially symmetrical pattern and later became confined to the ventral midline area, which runs axially through the germ band. RNA interference-mediated depletion of the spider sog gene led to a nearly complete loss of ventral structures, including the axial ventral midline and the central nervous system. This defect appeared to be the consequence of dorsalization of the ventral region of the germ band. By contrast, the extra-embryonic area formed normally. Furthermore, we showed that embryos depleted for a spider homologue of dpp failed to break the radial symmetry, displaying evenly high levels of sog expression except in the posterior terminal area. These results suggest that dpp is required for radial-to-axial symmetry transformation of the spider embryo and sog is required for ventral patterning. We propose that the mechanism of spider ventral specification largely differs from that of the fly. Interestingly, ventral specification in the spider is similar to the process in vertebrates in which the antagonism of Dpp/BMP signaling plays a central role in dorsal specification.

KEY WORDS: Spider, Embryogenesis, dpp, sog, Antagonist, Body axis formation, RNAi

INTRODUCTION

The fertilized eggs of Xenopus, as well as those of many other vertebrates, are transformed from a radially symmetrical form to a bilaterally symmetrical form with an axis (Gerhart, 2004; Kimelman and Bjornson, 2004). The dorsal lip of the blastopore in the amphibian embryo, known as Spemann’s organizer, can organize surrounding cells to form a complete body axis (Spemann and Mangold, 1924). This organizer itself contributes to the axial (dorsal-most) mesoderm becoming the notochord, concomitant with the patterning of the more ventral mesoderm and neural tissues. Molecular studies have suggested that the organizing activity is exerted by antagonizing bone morphogenetic protein (BMP) and Wnt signals (Piccolo et al., 1996; Harland and Gerhart, 1997; De Robertis et al., 2000). Chordin, noggin and follistatin are BMP antagonists expressed at the organizer (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Fainsod et al., 1997). Simultaneous depletion of these three BMP antagonists leads to a gross failure in the development of dorsal structures, including the notochord and neural tissues (Khokha et al., 2005).

In the fruit fly Drosophila melanogaster, a homologue of vertebrate BMP2/4, Decapentaplegic (Dpp), and a homologue of chordin, Short gastrulation (Sog), have also been shown to function antagonistically in dorsoventral (DV) pattern formation of the embryo (Irish and Gelbart, 1987; Padgett et al., 1987; Ferguson and Anderson, 1992a; Ferguson and Anderson, 1992b; François et al., 1994). Sog is the only Dpp antagonist that is known to function in the early Drosophila embryo, and two different roles for the molecule have been identified. The first role is to prevent the Dpp signal from invading the neuroectoderm that forms at the ventrolateral region (François et al., 1994; Biehs et al., 1996). The second role is to contribute to the formation of a sharp stripe of Dpp signaling activation at the dorsal-most region that will become the extra-embryonic amnioserosa (Ashe and Levine, 1999; Decotto and Ferguson, 2001). Although the latter role is considered to be unique to the fly, the former role is markedly similar to that of BMP antagonists in the development of vertebrate dorsal structures. This similarity has raised the hypothesis that the orientation of the Drosophila DV axis is opposite to that of the vertebrate DV axis, assuming that these axes had a common origin (Holley et al., 1995; De Robertis and Sasai, 1996; Ferguson, 1996; Holley and Ferguson, 1997; Bier, 1997). Despite the fascinating implications this would have for DV axis evolution, however, the null mutation for Drosophila sog only slightly reduces the neuroectoderm and barely affects the differentiation of the ventral midline (Fig. S1 in supplementary material) (François et al., 1994) not validating the potential importance of Dpp/BMP antagonism in DV axis specification in the common ancestor of Drosophila and vertebrates. The only organism other than Drosophila and vertebrates in which a Dpp/BMP antagonist has been studied is the ascidian, in which function of the antagonist has not been related to DV axis development or neural induction (Darras and Nishida, 2001).

In the phylum Arthropoda, twinned embryos can be produced spontaneously (see Fig. S2 in the supplementary material) or experimentally in spiders, horseshoe crabs and short-germ insects (Holm, 1952; Sekiguchi, 1957; Seitz, 1970; Sander, 1976; Itow et al., 1991). These may indicate the existence of different modes of axis specification from that of Drosophila. A study of the Dpp-Sog system within Arthropoda would contribute to a better understanding of the potential importance of Dpp/BMP antagonism in DV axis specification.
understanding of DV axis evolution. Spiders and horseshoe crabs are chelicerate arthropods that are phylogenetically distant from the insect *Drosophila* (Friedrich and Tautz, 1995; Hwang et al., 2001; Giribet et al., 2001). In early development of spiders, such as *Achaearanea tepidariorum*, the future DV axis becomes predictable by the onset of directional movement of a cellular thickening called the cumulus, which is formed at the center of the radially symmetrical germ disc and then shifts centrifugally to the rim (Akiyama-Oda and Oda, 2003). Graft experiments using *Agelena labyrinthica* showed that the cumulus has the ability to induce a secondary body axis (Holm, 1952). The shift of the cumulus is followed by formation of the extra-embryonic area and rearrangements of the germ disc cells. These morphogenetic events transform the germ disc into the bilaterally symmetrical germ band. Our previous study showed that, in the spider *Achaearanea tepidariorum*, a cluster of the mesenchymal cells at the cumulus (CM cells) is the source of Dpp signals (Akiyama-Oda and Oda, 2003; Yamasaki et al., 2005). *At-dpp* dechorionated embryos, which exhibited gross malformations, could not be staged on the basis of morphological characteristics; instead, they were staged based on the time past stage 5.

### MATERIALS AND METHODS

#### Animals

The two spider species *Achaearanea tepidariorum* and *Pholcus phalangioides* were collected at Kyoto University (Kyoto, Japan) and in Takatsuki city (Osaka, Japan). Dried eggs of *Artemia franciscana* were purchased (Tetra). Developmental stages of *Achaearanea* embryos were determined according to previous studies (Akiyama-Oda and Oda, 2003; Yamasaki et al., 2005). *At-dpp* dechorionated embryos, which exhibited gross malformations, could not be staged on the basis of morphological characteristics; instead, they were staged based on the time past stage 5.

#### cDNA cloning

We cloned *Achaearanea sog* (At-sog), *Pholcus sog* (Pp-sog), *Artemia sog* (Af-sog), and the *Achaearanea* genes single minded (At-sim), prospero (At-pros), engrailed (At-en) and optomotor blind (At-omb). Details of cDNA cloning are presented in Table 1. The sequences are available from the DNA Data Bank of Japan with the following accession numbers: *At-sog*, AB236147; *Pp-sog*, AB236148; *Af-sog*, AB236149; *At-sim*, AB236150; *At-pros*, AB236151; *At-en*, BAD01489; *At-omb*, AB177876. To characterize the deduced amino acid sequences of the cloned genes, BLASTP, BLAST 2 sequences (http://www.ncbi.nlm.nih.gov/blast/), PHYLIP version 3.5 and HarrPlot 2.0 (GENETYX Mac version 12) were used. BLASTP search revealed that the sequences of *At-Pros* and *At-En* are very close to those of *Cs-Pros* (Weller and Tautz, 2003) and *Cs-En* (Damen et al., 1998), respectively, which were previously reported in another spider species *Cupiennius salei*. Molecular phylogenetic trees were constructed for *At-Sim* and *At-Omb* (see Fig. S3 in the supplementary material).

#### Staining of embryos

*Achaearanea* and *Pholcus* embryos and *Artemia* larvae were fixed as described previously (Akiyama-Oda and Oda, 2003; Oda et al., 2005). Whole-mount in situ hybridization was performed as described previously (Lehmann and Tautz, 1994; Akiyama-Oda and Oda, 2003). For single-staining, digoxigenin (DIG)-labeled probe was used. For double-staining, embryos were incubated with a mixture of DIG- and fluorescein-labeled probes. The DIG-labeled probe was visualized in the same way as the single staining, followed by post-fixation and subsequent inactivation of alkaline phosphatase with 0.1 M glycine-HCl (pH 2.2). Then, the fluorescein-labeled probe was visualized using an alkaline phosphatase-conjugated rabbit anti-FITC antibody (DAKO, 1:200 dilution) and INT/BCIP solution (Roche). For nuclear staining, DAPI (Sigma) was used. To detect phosphorylated Mothers against dpp (pMad) protein for visualizing nuclei of cells responding Dpp signals, the PS1 antibody (Persson et al., 1998) was used as described (Akiyama-Oda and Oda, 2003). For sectioning, stained *Achaearanea* embryos were dehydrated in a graded ethanol-xylene series, embedded in TissuePrep (Fisher Scientific), and then serially sectioned at 5 μm.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’-3’)*</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sog1</td>
<td>tggcayccttytynccncnc</td>
<td>WHHFPVPP</td>
</tr>
<tr>
<td>sog2</td>
<td>gcrgcyctttrcarknc</td>
<td>(A/D)CEKCAQ</td>
</tr>
<tr>
<td>sog3</td>
<td>gatyngnnccntytytgg</td>
<td>DGLPFPG</td>
</tr>
<tr>
<td>sog4</td>
<td>acncknckcannncrcrna</td>
<td>CGAAYVYD</td>
</tr>
<tr>
<td>sog5</td>
<td>tggynmrathaaaryaaaraggnt</td>
<td>C1K/RINXK(D/E/C)</td>
</tr>
<tr>
<td>sog6</td>
<td>ccnggrcngtgytccrrca</td>
<td>CCKTCPP</td>
</tr>
<tr>
<td>sim1</td>
<td>gntqagtgytqntngc</td>
<td>RMKCVLAK</td>
</tr>
<tr>
<td>sim2</td>
<td>tanswytgncaanacanac</td>
<td>WWVQOSY</td>
</tr>
<tr>
<td>sim3</td>
<td>cnsowrcartdatneyatg</td>
<td>KVHCASG</td>
</tr>
<tr>
<td>pros1</td>
<td>aargcnaaryntgytgytgg</td>
<td>KAKLMMF(W/Y)</td>
</tr>
<tr>
<td>pros2</td>
<td>gctgtrgknrcwraytgytbc</td>
<td>EUK(YF)ARQA</td>
</tr>
<tr>
<td>en1</td>
<td>tggcngcntgigntwtytgg</td>
<td>WPWAVFY(W/F)</td>
</tr>
<tr>
<td>en2</td>
<td>trtnarcnctgytngcat</td>
<td>MAQGLYN</td>
</tr>
<tr>
<td>en3</td>
<td>aakgakatmgkttttckgaga</td>
<td>NTQ(D/E)INRULYTE</td>
</tr>
<tr>
<td>en4</td>
<td>trtttygradaacadtayt</td>
<td>KIWONFK</td>
</tr>
<tr>
<td>omb1</td>
<td>ayggnmmgnmgnatgytcc</td>
<td>NGRRMFMP</td>
</tr>
<tr>
<td>omb2</td>
<td>aangyngttngraangttrt</td>
<td>NPKAFK</td>
</tr>
</tbody>
</table>

*dis, a, gort, his, a, c, ort, kis gort, m, ao r, c, his a, c, ort, r, is a r, g, s, cor g, wi s, a, r, is, o, r, c.*

*Achaearanea sog* (At-sog) was amplified using the sog1 and sog2 primers, and *Pros* (Ps-sog) were amplified using the pros1 and pros2 primers. *Achaearanea en* (At-en) was amplified using the en1 and en2 primers for first PCR and the en3 and en4 primers for nested PCR. *Achaearanea sim* (At-sim) was amplified using the sim1 and sim2 primers for first PCR and the sim1 and sim3 primers for nested PCR. *Achaearanea pros* (At-pros) was amplified using the pros1 and pros2 primers. *Achaearanea en* (At-en) was amplified using the en1 and en2 primers for first PCR and the en3 and en4 primers for nested PCR. *Achaearanea omb* (At-omb) was amplified using the omb1 and omb2 primers. The PCR conditions were as follows: 1 cycle of 95°C for 5 minutes, 50°C for 2 minutes 30 seconds, 72°C for 40 seconds; 35 cycles of 95°C for 40 seconds, 72°C for 40 seconds; 1 cycle of 72°C for 10 minutes; and then a 4°C soak. For amplification of *At-en* and *At-omb*, the annealing temperatures were 45°C and 40°C, respectively, instead of 50°C. To obtain full-length cDNAs for *At-sog*, *At-omb* and *Artemia sog* cDNA libraries (Akiyama-Oda and Oda, 2003; Oda et al., 2005) were screened with DIG-labeled DNA probes for the PCR-amplified fragments. To obtain full-length cDNAs for *At-sim*, *At-pros* and Ps-sog, 5' and 3' RACE were performed using SMART RACE cDNA amplification kit (Clontech) and ExTaq polymerase (TaKaRa).
**RT-PCR**
Semi-quantitative RT-PCR was performed to compare the levels of gene expression at stage 9 (for At-sog dsRNA injected and non injected), or stage 5 (for At-dpp dsRNA injected and non injected) embryos. Total RNA was extracted from 30 eggs of each type using a MagExtractor RNA kit (Toyobo). The total RNA was treated with DNaseI (Stratagene), and a first-strand cDNA was prepared using an oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). The cDNA was used as a template for PCR reactions. The PCR conditions were as follows: 20 or more cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The primer sets used are as follows: for At-sog in At-sog cDNA experiments, tagataagggagaga and tgttccgtatgcctctgt; for At-sog in At-dpp RNAi experiments, aagttgcatgtaacatg and gttgcgtccttgctct; for At-sim, taggtaagcagaaatct and agggtaagcaagct; for At-dpp, ttagcatcacaagggaaag and gaccttgctcctatgagg; for histone H3, tacaacagacaagccctag and cttctccgtccttgtaat; for EF1α, tggcacaatgaaacac and tctgacagggattga. The sequences for histone H3 and EF1α were designed according to data from our Achaearanea EST project (unpublished results).

**RESULTS**

**Cloning of sog homologues**
We have cloned sog homologues from the spiders Achaearanea tepidariorum and Pholcus phalangioides, and the brine shrimp Artemia franciscana. The predicted amino acid sequences of these genes are comparable to those of Drosophila sog and vertebrate chordin through the entire length (Fig. 1). Four cysteine-rich domains, characteristic of Sog/chordin, are present at the expected positions in the spider and Artemia sequences. Therefore, the cloned genes were designated At-sog, Pp-sog and Af-sog, respectively.

**Deletion of At-sog expression by RNA interference**
To investigate the function of At-sog, we depleted At-sog expression from early spider embryos by parental RNA interference (pRNAi). Adult females were repeatedly injected with dsRNA corresponding to a 706-bp region (nt 334-1039) of At-sog cDNA, and their eggs were examined. In control experiments, dsRNA for gfp was used. Embryos derived from females injected with At-sog dsRNA, but not those from females injected with gfp dsRNA, showed abnormal development of limbs in the prosomal region (Fig. 4A-C). Time-lapse microscopy of live embryos and DNA staining of fixed embryos revealed that the abnormalities were due to lack of separation of extending limb buds in the individual segments (see **Fig. 1. The deduced amino acid sequences of At-sog, Pp-sog and Af-sog display high similarity to Drosophila Sog and vertebrate chordin.** (A) HarPlot analysis of the entire amino acid sequences of At-sog and Drosophila Sog (Dm-Sog). The parameter 'unit size to compare' was 8 and the parameter 'dot plot scores' was 2. The four cysteine-rich domains characteristic of Sog/chordin (CRs 1-4) are indicated by black bars. (B) Amino acid sequence comparisons of At-sog and the Sog/chordin protein of Pholcus (Pp), Artemia (Af), Anotheles (Ag), Drosophila (Dm) and Xenopus (Xl). Percentage identity and similarity (parentheses) were calculated for each of the CR-1 domains and the region intervening between the CR1 and CR2 domains (INT) using the BLAST2 sequences tool. Accession numbers of the proteins used are as follows: Ag-Sog, EAA06317.3; Dm-Sog, AAA89117.1; Xl-chordin, AAC42222. **Radial-to-axial shift of At-sog expression**
We examined expression patterns of At-sog transcripts by whole-mount in situ hybridization. At-sog transcripts were first detected at stage 5, when the cumulus shifts from the center to the rim of the germ disc (Akiyama-Oda and Oda, 2003). The signals appeared in the germ disc as a ring (Fig. 2A). This ring of At-sog expression was broken by the insertion of an At-sog-negative area (Fig. 2B), which corresponded to the presumptive extra-embryonic area as revealed by double staining for At-sog transcripts and pMad (Fig. 2J’). During stages 6 and 7, when the extra-embryonic area is expanded and the germ disc cells are dynamically rearranged, a large part of the embryonic area, excluding the forming caudal lobe and the periphery of the embryonic area, expressed At-sog (Fig. 2B-E). As the germ band formed and extended, the At-sog expression domain progressively narrowed (Fig. 2F). This narrowing At-sog expression domain was complementary to the pMad expression domain expanding from the dorsal side (Fig. 2K,L,L’). Eventually, the At-sog expression was confined to the ventral midline area (Fig. 2G). Sectioning revealed that At-sog staining was localized at the surface ectoderm, with no signal detectable in the mesodermal layer (Fig. 2H,I). Although the cells at the ventral midline area were morphologically indistinguishable from the neighboring ectoderm cells at this stage, this ventral midline area was characterized by specific expression of At-fork head (At-fkh) (Akiyama-Oda and Oda, 2003) and a sim homologue (At-sim) (Fig. 2M,N). The onset of At-fkh expression at the ventral midline area was at late stage 7, whereas the onset of At-sim expression was at stage 9. The expressions of At-sog, At-fkh, and At-sim were initially observed in nearly the same cell population (Fig. 2M,N). Later, however, different patterns of expression became evident (Fig. 3). This appeared to reflect the differentiation of several cell types within the ventral midline area.

The At-sog expression domain expanded anterior to the At-orthodenticle (At-otd) (Akiyama-Oda and Oda, 2003) expression domain (Fig. 2O,P). The At-sog expression domain also expanded posteriorly in accordance with the production of segments in the growing opisthosomal region (Fig. 2G,L), although the caudal lobe did not express At-sog. The axial pattern of At-sog expression in the germ-band stage embryo appeared to result from expansion of the expression along the emerging anterior-posterior (AP) axis and reduction of the expression along the emerging DV axis in accordance with dynamic cell rearrangements converting the germ disc to the germ band.

Similar to At-sog, Drosophila sog shows restricted expression at the ventral midline in the germ-band stage embryo (François et al., 1994). Similar ventral midline expression was also observed for Pp-sog (Fig. 2Q,R) and Af-sog (Fig. 2S,S’). The ventral midline expression of sog genes may be a conserved feature of the phylum Arthropoda.
The phenotypes of the abnormal embryos were classified as 'severe' and 'mild' based on the number of segments bearing unseparated limb buds. Such abnormal embryos were first found in the second or third round of egg laying after the first dsRNA injection (Fig. 4F-M). Whole-mount in situ hybridization revealed that, in the embryos exhibiting severe phenotypes, the level of *At-sog* mRNA was drastically reduced compared to the control embryos (Fig. 4D,E). A specific reduction in *At-sog* expression caused by pRNAi treatment was also confirmed by RT-PCR (Fig. 4Q). In addition, injection of dsRNA synthesized using another section of the *At-sog* cDNA (nt 1947-2987) also resulted in unseparated limbs (data not shown). The phenotypes obtained with injection of *At-sog* dsRNA were different from those obtained with injection of *At-dpp* dsRNA (see below), suggesting gene-specific effects. Taken together, these data suggest that pRNAi works in *Achaearanea* to deplete the expression of specific genes, as has been reported in other animal species (Fire et al., 1998; Bucher et al., 2002; Liu and Kaufman, 2003; Mito et al., 2005). Embryos in which *At-sog* expression was depleted by pRNAi, designated *At-sog* RNAi embryos hereafter, were further analyzed to understand the role of *At-sog* in the early development of the spider.

**Loss of ventral structures in *At-sog* RNAi embryos**

In *At-sog* RNAi embryos, the cumulus appeared and shifted, followed by the formation of the extra-embryonic area and the conversion of the germ disc to the germ band (Fig. 5A, compare with Fig. 7A). No apparent defects were found before or during these
processes (see Movie 1 in the supplementary material). A marked difference between At-sog RNAi and untreated embryos was first detected in the pattern of pMad expression at the germ-band stage (Fig. 5B,C). In At-sog RNAi embryos, the embryonic area was overwhelmed by nuclear pMad. In older At-sog RNAi embryos exhibiting extremely severe phenotypes, the ventral midline expression of At-sim and At-fkh was almost entirely missing (Fig. 5D,F, not shown for At-fkh); only low levels of expression that were coincident with persistent At-sog RNA at the posterior terminal area were detected. The reduction of At-sim expression in stage 9 At-sog RNAi embryos was confirmed by RT-PCR (Fig. 4Q). In At-sog RNAi embryos exhibiting milder phenotypes, spots of At-sim and At-fkh expression were occasionally observed in the prosomal region (Fig. 5E arrow, not shown for At-fkh). These spots were always located between separated limb buds and coincided with persistent expression of At-sog.

In Achaearanea, the central nervous system (CNS) develops from the areas adjacent to the At-sog-expressing ventral midline area. RNA staining of late stage 9 embryos for a homologue of pros, designated At-pros, visualized the developing neural cells (Fig. 5G), which were distributed in a pattern similar to that described for another spider species, Cupiennius salei (Stollewerken et al., 2001; Weller and Tautz, 2003). The dorsal area of each limb-bearing hemisegment is subdivided into a limb and a non-limb section. One

At-pros-positive cell cluster, tentatively called the dorsal-most cluster, was present at a regular position in each non-limb section (Fig. 5G arrows). In At-sog RNAi embryos exhibiting very severe phenotypes, the At-pros-positive neural cell clusters were mostly missing except for a few regularly aligned clusters located at similar AP positions to those of the dorsal-most clusters (Fig. 5G,1). In At-sog RNAi embryos exhibiting milder phenotypes, however, At-pros-positive cell clusters formed around the sites where At-sog was persistently expressed (Fig. 5H). These results suggest that the severe phenotypes caused by At-sog pRNAi were due to an almost complete loss of the ventral structures, including the ventral midline area and the CNS. The milder phenotypes generated by At-sog RNAi treatment imply that the ventral midline and the CNS form concomitantly by a mechanism requiring At-sog activity.

A homologue of omb, designated At-omb, was specifically expressed at the dorsal side of each limb bud that had started to extend (Fig. 5J), as described for Cupiennius omb (Prpic et al., 2003). In At-sog RNAi embryos exhibiting severe phenotypes, the domains of At-omb expression were fused to cover the entire width of the germ band (Fig. 5L). Similar alterations in the expression pattern were observed in the opisthosomal region (Fig. 5K,M). As shown previously, At-twist (At-twi) is expressed in mesodermal cell populations (Yamazaki et al., 2005). During stage 7, At-twi-expressing mesodermal cells become segmentally arranged in the prosoma. During later stages, additional stripes of At-twi-expressing cells appear in the opisthosoma. Each stripe of At-twi-expressing cells initially displays little unevenness along the predictable DV axis and then dorsally splits into two separate clusters at the sites of appendage formation (Fig. 5N,O), with At-twi-expressing cells becoming absent in the ventral areas. However, in At-sog RNAi embryos, in which the At-twi-expressing mesoderm developed normally until at least stage 7 (not shown), no dorsal separation of the mesodermal cells was observed in the prosoma or opisthosoma (Fig. 5P,Q). Taken together, these data indicated that the loss of the ventral structures caused by At-sog pRNAi appeared to result from gross dorsalization of the germ band.

**Persistent radial symmetry in At-dpp RNAi embryos**

Next, we conducted pRNAi experiments for At-dpp, which is initially expressed in the CM cells and later expressed in the dorsal region of the germ band where limb buds are formed (Akiyama-Oda and Oda, 2003). A specific reduction in At-dpp expression caused by the pRNAi treatment was confirmed by RT-PCR, although a very small amount of At-dpp transcripts was still detectable (Fig. 6I). In At-dpp RNAi embryos, the cumulus appeared and shifted normally (Fig. 6). However, the At-dpp RNAi embryos began to exhibit defects from the beginning of stage 6, when the extra-embryonic area starts to differentiate. DNA staining revealed that the extra-embryonic area, which can be easily recognized by the sparse distribution of nuclei (Fig. 7A), was reduced in size (Fig. 7B) or lost (Fig. 7C). The defective germ disc was extended longitudinally to envelop the entire yolk mass. Consequently, in severe cases, morphologically monotonous embryos were formed with little asymmetry (Fig. 6, Fig. 7C). Staining of such At-dpp RNAi embryos for a homologue of en, designated At-en, revealed rings of At-en expression instead of the two-ended bands of At-en expression observed in normal embryos (Fig. 7D-F). Similarly, a ring of expression was observed for At-otd in severely defective At-dpp RNAI embryos (Fig. 7G-I). These persistent circular patterns of gene expression probably reflected the loss of the extra-embryonic area. Striped expression of At-en (Fig. 7E) and anterior and posterior...
expression of At-otd and At-caudal (Akiyama-Oda and Oda, 2003), respectively, in At-dpp RNAi embryos (Fig. 7G-L) imply that At-dpp RNAi had little effect on AP patterning, although there were fewer At-en stripes than normal.

Furthermore, we examined At-sog expression in At-dpp RNAi embryos at different stages. RT-PCR showed that there was no detectable difference in the level of At-sog transcripts between untreated and At-dpp RNAi embryos at late stage 5 (Fig. 6f). Expression patterns of At-sog transcripts were observed in more than 29 late stage 5 embryos derived from the egg sac that was used for the RT-PCR experiment. It was found that all of the embryos showed more or less reduced asymmetry of the At-sog expression pattern (not shown), but none displayed complete symmetry, indicating that At-sog transcription was still affected by the shifting cumulus even in the At-dpp RNAi embryos. However, in severely defective At-dpp RNAi embryos at the later stages (stages 8 and 9) the entire surface ectoderm except for the posterior terminal area expressed At-sog transcripts at evenly high levels (Fig. 7M-O). Little asymmetry was recognized in the At-sog expression pattern. At-dpp RNAi embryos at the later stages were further examined for genes whose expression patterns reflect differences along the DV axis in the normal germ band. Staining for At-twi showed rings of At-twi-expressing mesodermal cells (Fig. 7P-R). No significant staining for At-pros, At-sim, or At-omb was obtained (Fig. 7S; not shown for At-sim and

Fig. 4. Depletion of At-sog by dsRNA injection results in unseparated limb buds. (A-C) Flat preparations of DNA-stained stage 9 embryos derived from females injected with At-sog dsRNA (A,B) or gfp dsRNA (C). The limb bud defects were classified as severe (A) and mild (B) (see Materials and methods). (D,E) Comparison of At-sog expression by whole-mount in situ hybridization in stage 9 embryos derived from females injected with At-sog (D) or gfp (E) dsRNA. (F-P) Time course of phenotype expression in limb buds following dsRNA injection. Each graph refers to one female injected with At-sog (F-M) or gfp (N-P) dsRNA. Each vertical bar indicates the relative numbers of embryos exhibiting severe (red), mild (yellow), and normal (blue) phenotype in each egg sac. Unfertilized eggs or embryos exhibiting non-specific defects before germ band formation were not considered. The day when the first dsRNA injection was performed was defined as day 0. The numbers in parentheses indicate how many times dsRNA injection was performed. Asterisks indicate unhealthy egg sacs (see Materials and methods). Eggs from the egg sacs labeled D,E,Q in graphs F and N were used for the experiments shown in D,E,Q, respectively. (Q) RT-PCR comparison of the levels of At-sog, At-sim, ef1α and histone H3 transcripts in At-sog RNAi (sog Ri) and untreated (u.t.) embryos at stage 9. ‘+’ and ‘−’ indicate reactions with and without reverse transcriptase, respectively. Note that faint bands of At-sog and At-sim transcripts are visible in the ‘+’ lanes of the At-sog RNAi sample. Ch, chelicerae; Pp, pedipalps; L1-L4, first to fourth walking legs; sog Ri, At-sog RNAi, gfp Ri, gfp RNAi. Scale bars: 100 μm.
At-omb). In addition, the patterns of At-fkh expression, which varied among embryos, were radially symmetrical (not shown). Taken together, these data suggested that the At-dpp-depleted embryos were prevented from breaking the radial symmetry.

**DISCUSSION**

**Roles of spider Sog and Dpp in axis specification**

In this study, we have molecularly dissected how the embryo of the spider *Achaearanea tepidariorum* establishes the axial symmetry during development (Fig. 8). Our data suggest that Dpp signaling is essential to specify the extra-embryonic area at the future dorsal side in the germ disc (Fig. 7), and that the subsequent antagonism of Dpp signaling by Sog appears to specify the ventral-most and flanking domains, which will become the ventral midline and the CNS (Fig. 5).

Depletion of At-dpp prevented the embryo from breaking the radial symmetry, therefore, the At-Dpp-mediated specification of the extra-embryonic area is crucial for radial-to-axial symmetry transformation of the spider embryo (Fig. 8, yellow). Despite the asymmetric patterns of At-sog expression from late stage 5 onward (Fig. 2), At-sog function appears not to be involved in the formation of the extra-embryonic area or the germ band (Fig. 5A). However, our data cannot exclude the possibility that the suppressed At-sog is sufficient to play a role in the early events. Since we failed to observe At-dpp RNAi embryos showing radially symmetrical At-sog expression at late stage 5, it remains unclear whether At-dpp is needed to make the initial At-sog expression asymmetric. The ubiquitous expression of At-sog transcripts at high levels in the later At-dpp RNAi embryo (Fig. 7N) suggested that At-sog transcription is negatively regulated by Dpp signaling. The gradual expansion of the pMad-positive area from the dorsal side (Fig. 2K,L) might be achieved by the combination of positive feedback loops of Dpp signaling, as proposed in *Drosophila* (Biels et al., 1996), antagonism of Dpp signaling by Sog and progressive repression of At-sog transcription by the Dpp signals.

The patterning defects in the At-sog RNAi embryo were consistent with the At-sog expression being confined to the ventral midline area in the normal embryo (Figs 2, 5). One of the most intriguing issues in understanding the At-sog-mediated mechanism of ventral specification is how At-sog expression persists at the presumptive ventral midline area to trigger specific gene expression. In the mild At-sog RNAi phenotype, neural marker expression was

---

**Fig. 5. The germ bands are dorsalized in sog RNAi embryos.** (A) An At-sog RNAi embryo at stage 8 stained for DNA and At-otd transcripts. (B,C) Ventral views of untreated (B) and At-sog RNAi-treated (C) stage 8 embryos stained for pMad. (D-F) Flat preparations of untreated (D) and At-sog RNAi (E,F) stage 9 embryos stained for At-sog (red) and At-sim (purple). The arrow in E indicates the L1 segment, in which a patch of At-sog and At-sim expression intervenes between separated limb buds. (G-I) Flat preparations of untreated (G) and At-sog RNAi (H,I) late stage 9 embryos stained for At-sog (red) and At-pros (purple). Arrows in G indicate dorsal-most neural cell clusters. (J-Q) Prosomal (J,L,N,P) and opisthosomal (K,M,O,Q) regions of untreated (J,K,N,O) and At-sog RNAi (L,M,P,Q) stage 9 embryos stained for At-omb (J-M) or At-twi (N-Q) transcripts. a, anterior; p, posterior; Ch, chelicerae; Pp, pedipalps; L1-L4, first to fourth walking legs; sog Ri, At-sog RNAi. If not specified, anterior is to the top. Scale bars: 100 µm.
observed around patches of persistent At-sog expression (Fig. 5H). This observation suggests that neural development depends on At-sog expression rather than position in the germ band.

How is the AP axis specified in the Achaearanea embryo? In the germ disc, although its center is presumably determined to be the posterior pole, every point at its rim may have the potential to become the anterior pole (Akiyama-Oda and Oda, 2003). The germ-disc stage embryo has an axis that is probably equivalent to the animal-vegetal axis in other phyla, but does not yet have the AP axis specified. AP axis specification is considered to involve two processes; one is development (or refinement) of AP positional information, and the other is specification of the anterior pole. The former, which was little affected in At-dpp RNAi embryos (Fig. 7), probably does not depend on Dpp signals, but the latter does depend on Dpp signals. In the normal germ disc, an anterior pole is predicted to be the most distant point from the extra-embryonic area induced by Dpp signals (Akiyama-Oda and Oda, 2003). This rule may be extended to account for duplicated AP axes caused by grafting of the cumulus in Holm’s experiments (Holm, 1952). In this experimental situation, two anterior poles are predictable at the halfway points along the germ disc rim between the native and ectopic extra-embryonic areas. The later At-dpp RNAi embryo had a recognizable anterior pole despite lacking the extra-embryonic area (Fig. 7C). However, this anterior pole simply resulted from symmetric longitudinal extension of the germ disc, the periphery of which converged at the pole of the yolk side. In addition, the longitudinal extension of the At-dpp RNAi germ disc implies that Dpp signaling may not be necessary to induce the dynamic cell rearrangements during germ band formation.

Comparison of axis specification between fly and spider
There are two major differences in the roles of Drosophila and Achaearanea Sog. First, fly Sog is necessary for specifying the extra-embryonic area (Ashe and Levine, 1999; Decotto and Ferguson, 2001) but spider Sog probably is not (Fig. 5A). Second, spider Sog (Fig. 5D-I) but not fly Sog (François et al., 1994) (see Fig. S1 in the supplementary material) is essential for specifying the ventral tissues that run axially through the germ band. These differences may reflect evolutionary changes in the gene regulatory networks.

Fig. 6. The At-dpp RNAi embryos exhibit defects in formation of the extra-embryonic area and germ band. (A-E,A’-E’) Time course of a developing At-dpp RNAi embryo. (F-H,F’-H’) Time course of a developing normal embryo. Images were taken at the time points indicated (day: hour: minute). For both embryos, time point 0:00:00 is adjusted to mid-stage 5, when the cumulus (arrows) is midway through shifting. The images in A-E, F-H show posterior views (or the top of the germ disc), and those in A’,B’,F’,H’ show lateral views with the posterior to the bottom. The DV orientation of the embryo shown in C’-E’ could not be determined, but the posterior side is to the bottom. Arrows in A,A’,B’,B’,F,F’ indicate the cumulus, and arrowheads in G,G’,H indicate the expanding extra-embryonic area (ex) in the normal embryo. Since the cells at the extra-embryonic area are very thin, the yolk mass is seen through them. By contrast, the embryonic area or the germ band (gb) is seen as opaque (or white) material. Note that the extra-embryonic area is not formed in the At-dpp RNAi embryo (C-E,C’-E’). (I) RT-PCR comparison of the levels of At-dpp, At-sog, ef1a and histone H3 transcripts in At-dpp RNAi (dpp Ri) and untreated (u.t.) embryos at stage 5. ‘+’ and ‘−’ indicate reactions with and without reverse transcriptase, respectively. Note that a faint band of At-dpp transcripts is visible in the ‘+’ lane of the At-dpp RNAi sample. Scale bar: 100 μm.
Axis specification in spider embryo

DV axis specification in the *Drosophila* embryo essentially relies on a nuclear gradient of the maternal transcription factor Dorsal, which simultaneously determines the differential expression domains of sog, dpp and many other genes (Roth et al., 1989; Ray et al., 1991; Stathopoulos and Levine, 2002; Stathopoulos and Levine, 2004). This fly system could have reduced the contribution of cell-cell interactions, explaining the relatively minor role of *Drosophila* Sog in ventral specification. However, the Dorsal gradient, which peaks at the ventral-most site, may have a lower resolution in the dorsal half than in the ventral half (Jiang and Levine, 1993). A Sog-mediated mechanism that sharpens the Dpp-activating domain at the dorsal-most region was recently proposed (Ashe, 2005; Shimmi et al., 2005; Wang and Ferguson, 2005). This mechanism might have been needed to compensate for the low resolution of the Dorsal gradient in this dorsal region.

In contrast to the Dorsal-based mechanism that organizes the DV axis in *Drosophila*, a mechanism controlling the direction of CM cell migration is important for initial DV polarity in *Achaearanea*, although its molecular basis remains unclear. As is evident from this study, the spider system appears to require a series of cell-cell interactions involving At-Dpp and At-Sog to initiate ventral-specific gene expression. These cell-cell interactions may well account for the regulative nature of body axis formation of spider embryos proposed by classical experiments (Holm, 1952; Sekiguchi, 1957; Seitz, 1970), although the mechanism of secondary axis induction in spiders remains to be studied. Based on the results obtained in the analyses of spider *dpp* and *sog*, we propose that one of the most fundamental differences in the mechanisms that pattern the early fly and spider embryos is ventral specification. This difference may reflect different degrees of contribution made by maternal determinants and zygotic cell-cell interactions.

In addition, the mesoderm originates from between two separate lines of presumptive ventral midline cells in the *Drosophila* embryo (Leptin, 2004). This situation is completely different from that of the *Achaearanea* mesoderm, which was suggested by At-twi expression patterns at stages 5 and 6 to have radially symmetrical origins at the peripheral and central areas of the germ disc (Yamazaki et al., 2005). In spider development, independent gene regulatory networks appear to specify the DV pattern and the mesoderm.

**Comparison of axis specification between vertebrates and spider**

Our discovery of the differences between the fly and spider provide an opportunity to rethink how arthropod embryos can be compared with vertebrate embryos from the viewpoint of developmental evolution. Interestingly, the spider situation in which sog activity is essential to specify the ventral domains is similar to the vertebrate situation in which the antagonism of Dpp/BMP signaling plays a central role in dorsal specification (De Robertis et al., 2000; Oelgeschläger et al., 2003; Khokha et al., 2005). The ventral midline area in the spider embryo is comparable to the presumptive notochord area in the vertebrate embryo in that both areas are the centers of the Dpp/BMP antagonism and adjoin the CNS. In addition, the axial expression of At-fkh and At-sim (Fig. 2M,N) is reminiscent of the expression of their homologues in chordate notochords (Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993; Strähle et al., 1993; Shimauchi et al., 1997; Shimeld, 1997; Terazawa and Satoh, 1997; Mazet and Shimeld, 2002). Whether the spider ventral midline is homologous to the vertebrate notochord is the emerging question. To satisfactorily answer this question, we will need to consider the reason why the arthropod ventral midline is ectodermal and the vertebrate notochord is mesodermal. The

---

**Fig. 7.** At-*dpp* RNAi embryos fail to break the radial symmetry. (A-C) DNA staining of untreated (A) and At-*dpp* RNAi (B,C) embryos at stage 8. All these embryos were also stained for At-otd transcripts. (D-R) Expression of At-en (D-F), At-otd (G-I), At-caudal (J-L), At-*sog* (M-O), and At-twi (P-R) transcripts in untreated (D,G,J,M) and At-*dpp* RNAi (E,F,H,I,K,L,N,O,Q,R) embryos at stage 9 (D-F,M-R) or stage 8 (G-L). D,G,J and P are lateral views and M is a ventral view; E,I,L,O,R are viewed from the directions indicated by arrows in E,H,K,N,Q. (S1,S2) Expression of At-*pros* transcripts in untreated (left in S) and At-*dpp* RNAi (right in S) late stage 9 embryos. The two boxed regions in S are magnified in S1 and S2. a, anterior; p, posterior; dpp Ri, At-*dpp* RNAi. Scale bars: 100 μm.
milder phenotypes of At-sog RNAi embryos (Fig. 5E,H) appeared to reflect concomitant development of the ventral midline and the CNS. It is intriguing to investigate similarities and differences in the mechanisms of neural induction in the spider and vertebrate embryos. Finally, the Achaearanea experimental system could contribute to a better understanding of the evolutionary relationships between the development of arthropod and vertebrate embryos.

We thank T. Tabata for the PS1 antibody, E. Bier for the Drosophila sog and sim cDNA clones, A. Stollewerk and D. Tautz for the anti-Cupenius Pros antibody, F. Matsuzaki for the MRF1 antibody, T. Niimi for instruction on dsRNA preparation, the Drosophila Bloomington stock center for the sog mutant line, and K. Agata and H. Tarui for sharing Achaearanea EST data before publication. We are grateful to M. Irie, M. Okubo, S. Okajima and A. Noda for technical assistance. We also thank G. Eguchi and members of the PRESTO research group of ‘Recognition and Formation’ for helpful discussion, and Sh. Tsukita and K. Nakamura for support.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/12/2347/DC1

Fig. 8. Schematic representation summarizing developmental events in the normal Achaearanea embryo and primary defects in the At-dpp and At-sog RNAi embryos. The state of the embryo symmetry is shown at the top. Time (hours; h) after egg laying at 25°C and the stages of development are also shown. Each developmental event takes place during the period indicated by the solid bar (faded areas indicate uncertain start or end points). In illustrations for the respective stages, the yolk area, the embryonic area, the extra-embryonic area and the limb buds are outlined. Stages 6 and 8 are shown in yellow and blue, respectively, when primary defects were observed in At-dpp RNAi and At-sog RNAi embryos (see the insides of the boxes). a, anterior; p, posterior; d, dorsal; v, ventral.

References
Axis specification in spider embryo


