

Tsukushi cooperates with VG1 to induce primitive streak and Hensen's node formation in the chick embryo

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Three classes of signaling molecule, VG1, WNT and BMP, play crucial roles in axis formation in the chick embryo. Although VG1 and WNT signals have a pivotal function in inducing the primitive streak and Hensen's node in the embryo midline, their action is complemented by that of BMP antagonists that protect the prospective axial tissue from the inhibitory influence of BMPs secreted from the periphery. We have previously reported that a secreted factor, chick Tsukushi (*TSK*), is expressed in the primitive streak and Hensen's node, where it works as a BMP antagonist. Here, we describe a new crucial function for TSK in promoting formation of the primitive streak and Hensen's node by positively regulating VG1 activity. We provide evidence that TSK directly binds VG1 in vitro, and that TSK and VG1 functionally interact in axis formation, as shown by biological assays performed in chick and *Xenopus* embryos. Furthermore, we show that alternative splicing of *TSK* RNA leads to the formation of two isoforms (*TSKA*, originally designated as *TSK*, and *TSKB*) that differ in their C-terminal region. Biochemical and biological assays indicate that *TSKB* is a much weaker BMP antagonist than *TSKA*, although both isoforms efficiently interact with VG1. Remarkably, although both *TSKA* and *TSKB* are expressed throughout the early extending primitive streak, their expression patterns diverge during gastrulation. *TSKA* expression concentrates in Hensen's node, a well-known source of anti-BMP signals, whereas *TSKB* accumulates in the middle primitive streak (MPS), a region known to work as a node-inducing center where VG1 expression is also specifically localized. Loss-of-function experiments demonstrate that *TSKB*, but not *TSKA*, function is required in the MPS for induction of Hensen's node. Taken together, these results indicate that TSK isoforms play a crucial role in chick axis formation by locally modulating VG1 and BMP activities during gastrulation.

KEY WORDS: Chick, Tsukushi, BMP antagonist, VG1, Primitive streak, Hensen's node

INTRODUCTION

The chick embryonic axis is formed during gastrulation in two consecutive steps: first, the primitive streak is induced and becomes recognizable as a thickening of cells at the posterior pole of the embryo. These cells move out along the midline during gastrulation to form a rod-like structure that extends approximately three-fifths the length of the embryo. In a second phase, while the primitive streak regresses in a posterior direction, the prospective mesendodermal progenitors ingress through the primitive streak inside the blastocoel to form the mesodermal and endodermal embryonic germ layers (reviewed by Bellaris, 1986). The anterior tip of the primitive streak is known both as Hensen's node and the organizer because of its ability to dorsalize the mesoderm and to induce and pattern the nervous system (Streit and Stern, 1999). Embryological studies have shown that formation of the primitive streak and the organizer is regulated by two key signaling centers that work at different stages during gastrulation. First, during pre-streak stage, the posterior marginal zone (PMZ), a small posterior domain within the marginal zone, has a crucial role in inducing adjacent epiblast cells to form the primitive streak. When this

domain is transplanted to an ectopic site of the marginal zone of a host embryo, it induces a secondary axis including the organizer (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989). Later in gastrulation, during primitive streak stages, a second signaling center located in the middle primitive streak (MPS) is involved in controlling induction of the organizer at the anterior tip of the primitive streak. Transplantation experiments have shown that this tissue, known as the 'node inducing center', is sufficient to induce adjacent cells to become organizer (Joubin and Stern, 1999). Remarkably, the inducing activities of both the PMZ and the MPS have been shown to be mediated by the same classes of signaling molecules, namely VG1, a TGF β superfamily member, and WNT proteins (Joubin and Stern, 1999). Grafts of VG1- and WNT-expressing cells in pre-streak embryos can reproduce the activity of the PMZ to induce primitive streak (Joubin and Stern, 1999), while VG1 and WNT1 misexpression during gastrulation can mimic the ability of the MPS to induce an ectopic organizer (Joubin and Stern, 1999). However, the action of VG1 and WNT signals needs to be complemented at both stages by mechanisms that locally lower the levels of BMP signaling, as members of this different class of TGF β molecules have been shown to have a very strong inhibitory activity towards primitive streak and Hensen's node formation (Streit et al., 1998).

We have previously described the isolation of chick Tsukushi (*TSK*), a soluble molecule belonging to the small leucine-rich proteoglycan (SLRP) family (Ohta et al., 2004). We showed that *TSK* is expressed in the primitive streak and in Hensen's node during chick gastrulation, where it works as a BMP antagonist. Previous gain- and loss-of-function experiments have indicated that *TSK* function is both sufficient and necessary to provide a BMP antagonistic activity required for induction of Hensen's node by the

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MPS (Ohta et al., 2004). Here, we report on novel observations that provide evidence that TSK controls formation of the chick embryonic axis by also regulating the activity of VG1. We show that alternative splicing of the chick Tsukushi gene generates two isoforms that differ in their C-terminal region. Remarkably, these two isoforms have different expression patterns during chick gastrulation and different biochemical activities. The *TSKA* isoform, which corresponds to the originally described TSK (Ohta et al., 2004), is a strong BMP antagonist, the expression of which becomes progressively restricted to Hensen's node and to the anterior primitive streak during gastrulation. By contrast, the newly identified *TSKB* isoform is a significantly weaker BMP antagonist than *TSKA*, which becomes enriched in the MPS. Both *TSKA* and *TSKB* directly bind to VG1 in vitro and show clear functional interactions with VG1 in facilitating embryonic axis induction both in *Xenopus* and chick embryos. Finally, loss-of-function experiments demonstrate that *TSKB*, but not *TSKA*, function is required in the MPS for induction of Hensen's node. Taken together, these experimental evidences provide considerable advance on our understanding of the molecular mechanism that controls embryonic axis formation in chick, by identifying TSK isoforms as crucial modulators of different branches of TGF β signaling during gastrulation.

MATERIALS AND METHODS

Embryos and culture

Fertile White Leghorn chicken embryos were staged as previously described (Eyal-Giladi and Kochav, 1976; Hamburger and Hamilton, 1951). Embryos were explanted in EC culture (Chapman et al., 2001). The expression vectors for *TSKA* (Ohta et al., 2004), *TSKB*, VG1 (Shah et al., 1997), Nodal (Bertocchini and Stern, 2002), FGF8B (Sato and Nakamura, 2004) or chordin (Streit et al., 1998) were transfected into COS-7 cells using LipofectAmine 2000 (Gibco-BRL). Forty-eight hours later, cell pellets containing 1000-2000 cells were generated by hanging drop cultures (Ohta et al., 1999). Rat B1-fibroblast cells secreting WNT1 were used as described (Shimizu et al., 1997) (a gift from J. Kitajewski). Cell aggregates were grafted to the indicated regions in the embryo using a micropipette. Embryos were incubated for different periods, fixed and processed for in situ hybridization. The experiments described here, like those of previous works in this field, rely on misexpression of soluble factors delivered by grafts of transfected cells.

Cloning and RT-PCR

The developing primitive streak was dissected from chick embryos at stage 2. Explants were taken from chick embryos at stage 3 and subdivided into the anterior primitive streak (PS-A) and the posterior primitive streak (PS-P). Explants from chick embryos at stage 4 were also taken and subdivided into the Hensen's node (HN), the PS-A, the middle primitive streak (PS-M) and the PS-P. Total RNA from each fraction was extracted with TRIZOL reagent (Invitrogen) and first-strand cDNA was synthesized using oligo d(T)₁₂₋₁₈ primer and Superscript II reverse transcriptase (Invitrogen). PCR was performed with the following primers: TSK-S (5'-ATGAATTCCGATGCAGTTCCTAGCCTGGTTCAAC-3') and TSK-AS (5'-ATCTC-GAGCGTGGGGTTTTGGACGGTTGAGGT-3'). PCR amplification (30 cycles) yielded a *TSKB* cDNA band of 1.5 kb. Excision of a gel slice at ~1.1 kb DNA size, followed by DNA elution and further PCR amplification (20 cycles) using the same primer set, produced a *TSKA* cDNA band of 1.1 kb. After subcloning and sequencing of the amplified cDNA fragments, two types of Tsukushi cDNAs were identified. We designated the original TSK as *TSKA* (Ohta et al., 2004) and the other as *TSKB* (GenBank Accession Number AB195969).

For RT-PCR of chordin and GAPDH, the following primers were used: *Chordin-S* (5'-GGCACCAGGGTGAAGTGCACCTGCG-3'); *Chordin-AS* (5'-GCGGCTCCATGCCTCTGCTGT-3'); *GAPDH-S* (5'-GGCTGCTA-AGGCTGTGGGGA-3'); *GAPDH-AS* (5'-TATCAGCCTCTCCACC-TCC-3').

In situ hybridization

In situ hybridization was performed as described (Ohta et al., 2004). Digoxigenin (DIG)-labeled antisense and sense RNA probes of *TSK*, *brachyury*, *gooseoid* and *chordin* were produced from their constructs subcloned into pBluescript II (Stratagene).

Immunoprecipitation

An immunoprecipitation (IP) assay was carried out as previously described (Ohta et al., 2004). Briefly, COS-7 cells were transfected with the following constructs: *Myc-His tagged TSKA* (Ohta et al., 2004), *Myc-His tagged TSKB*, *Myc-tagged VG1* (Shah et al., 1997), *Flag-tagged BMP7* (Ohta et al., 2004) and *Flag-tagged BMP4*. The conditioned medium containing the tagged proteins was incubated for 12 hours at 4°C. After incubation with ProBond resins (Invitrogen), the beads were washed with IP buffer without BSA or the same buffer with high salt (450 mM), 0.5% Triton-X-100 and 0.5% CHAPS. The bound proteins were subjected to SDS-PAGE and blotted onto a nylon membrane. Myc-tagged and Flag-tagged proteins were immunodetected using the antibodies 9E10 (DSHB) and M2 (Sigma), respectively.

Xenopus secondary axis formation

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For the secondary axis induction experiment, mRNA was injected into the ventro-vegetal midline at eight-cell stage embryo. The judgment of complete or incomplete secondary axis is as same as described by Lemaire et al., (Lemaire et al., 1995).

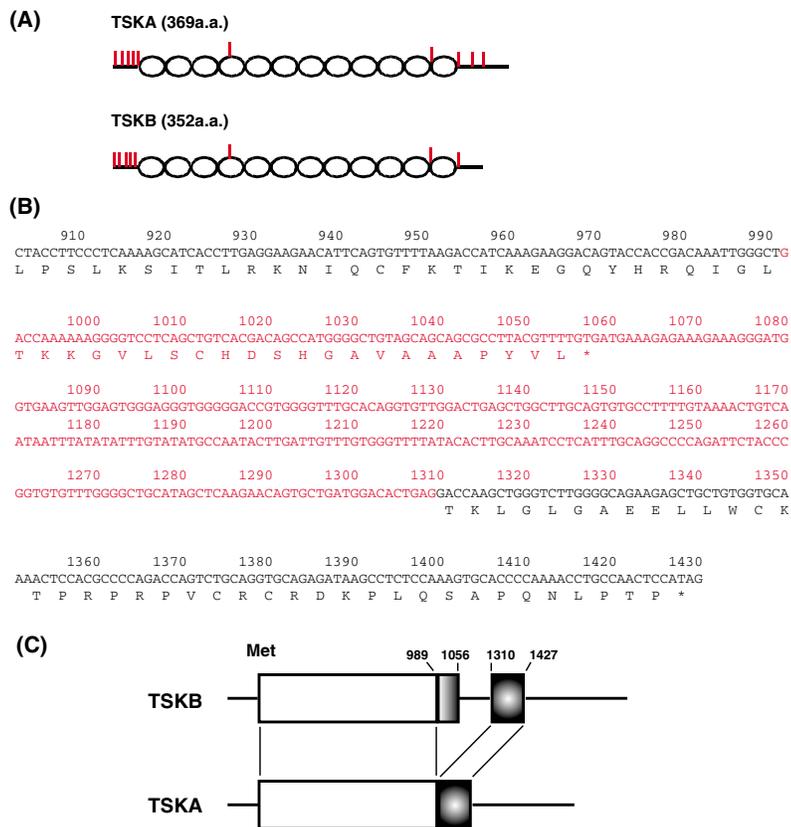
Electroporation of siRNA into chick embryos

The 'TSKs' siRNA (5'-CTCTCTGGAAGTCCTCCA-3') was used to silence the function of both *TSKA* and *TSKB*. *TSKA* siRNA (5'-GTCTGCAGGTGCAGAGATA-3') which silences the *TSKA* specifically (Ohta et al., 2004) and control siRNA; 5'-CAAGATCCAGAAGGTCGGT-3'. COS-7 cells were transfected with Myc-tagged *TSKA* or *TSKB* and with 2 μ g of control siRNA, *TSKA* or *TSKs* siRNA, and the amount of *TSKA* and *TSKB* proteins were analyzed as described above. For electroporation, 0.2 μ l siRNA solution (0.5 μ g/ μ l), including 0.05% Fast Green, was microinjected into the space between the vitelline membrane and the epiblast. The MPS was transfected by electroporation with two successive square pulses of 7 V and 50 ms using platinum square electrodes (2 mm \times 2 mm) 3.5 mm apart. The middle of the primitive streak, roughly 200 μ m \times 600 μ m, was excised from transfected donor embryos and implanted into the lateral position to Hensen's node of a host embryo. The embryos were incubated for 6 hours at 38.5°C and in situ hybridization was performed with *TSK*, *chordin* or *gooseoid* probe. To confirm the ability of *TSKs* siRNA in the chick, after electroporation, the node was excised and cultured for 6 hours at 38.5°C, and then the transfected MPSs were excised for RT-PCR using the following primers: TSK-S and TSK-AS1 (5'-ATACATGTT-CACAAAACGTAAGGCGC-3'); *GAPDH-S* and *GAPDH-AS*, VG1-S (5'-TAGAATTCAAGTGCTTACAACGTCCCGTC-3'); VG1-AS (5'-ATCTCGAGTCTGCAGCCACACTCATCCAC-3').

RESULTS

The TSK gene produces two isoforms by alternative 5' splicing

Chick Tsukushi (*TSK*) is a member of the SLRP family containing 12 leucine-rich repeats and two cysteine clusters located at the N and C termini (Ohta et al., 2004). Sequence alignment revealed extensive conservation of *TSK* sequences across different organisms, with the exception of *TSK* C-terminal region that appears to be more divergent from that of other *TSK* homologs (data not shown). We speculated that the *TSK* gene produces another isoform with higher conservation of the C-terminal region by alternative splicing. To test this possibility, we performed RT-PCR on RNA extracted from stage 4 chick embryos and found two types of *TSK* cDNAs (Fig. 1A). One of these cDNA corresponds to the previously isolated *TSK* cDNA (Ohta et al., 2004) and we re-designated it as *TSKA*. However, the second cDNA encodes for a new isoform of *TSK*, that we named *TSKB*, the C-terminal



sequence of which is conserved with respect to those of other TSK orthologs. A chicken genome database search (Ensembl Chicken Genome Browser) indicated that the *Tsukushi* gene is located on chromosome 1. Fig. 1B shows the alignment of *TSKB* cDNA and amino acid sequences at the C-terminal region. *TSKB* cDNA sequence is identical to that of the *Tsukushi* genomic DNA. In the *TSKA* isoform, nucleotides 990–1309 (red) are deleted by alternative 5' splicing. Fig. 1C schematize the alternative 5' splicing of TSK pre-RNA, resulting in the conversion of *TSKB* to *TSKA*.

Expression of *TSKA* and *TSKB* during early chick development

We could not distinguish the expression of *TSKA* and *TSKB* by in situ hybridization, because the *TSKA* cDNA does not have any specific regions that are not present in *TSKB*. Therefore, we performed RT-PCR to determine the precise spatiotemporal expressions of *TSKA* and *TSKB* during chick gastrulation. At stage 2, the emerging primitive streak expressed both *TSKA* and *TSKB* (Fig. 2A). At stage 3, during primitive streak elongation, we divided the primitive streak into anterior and posterior parts (PS-A and PS-P, respectively). Both *TSKA* and *TSKB* were expressed throughout the primitive streak (Fig. 2B). Chordin, which is expressed in the anterior tip of the developing primitive streak at this stage (Lawson et al., 2001), was predominantly expressed in the anterior fragment (Fig. 2B). At stage 4, when the primitive streak has reached its full extension, we divided the embryo axial structures into four parts, namely Hensen's node (HN), and the anterior, middle and posterior parts of the primitive streak (PS-A, PS-M and PS-P). *TSKA* expression was observed exclusively in the anterior part (HN and PS-A) of the embryonic axis, while *TSKB* was expressed throughout the axial structures with a peak of expression at the MPS. At this stage, chordin was specifically detected in Hensen's node (Lawson

et al., 2001; Skromne and Stern, 2002) (Fig. 2C). At all stages examined, *TSKB* was expressed at higher levels than *TSKA*, because with the same set of primers the *TSKB* isoform was detectable after a lower number of PCR cycles than was the *TSKA* isoform.

TSKB is a significantly weaker BMP antagonist than *TSKA*

We have previously shown that *TSKA* binds to BMP4 directly and inhibits its activity in vitro and in vivo (Ohta et al., 2004). To investigate whether *TSKB* can also work as a BMP antagonist, we initially performed overexpression assays in *Xenopus* embryos. Injection of 600 pg of *TSKB* mRNA into a ventral vegetal blastomere of eight-cell stage embryos (Fig. 3B) did not induce ectopic axial structures, although 400 pg of *TSKA* mRNA were very efficient at inducing a secondary axis (Fig. 3A) (Ohta et al., 2004). We next performed co-immunoprecipitation assays. When Myc-His-tagged *TSKA* or *TSKB* was reacted with Flag-tagged BMP4, immunoprecipitation with nickel-chelating resins pulled down BMP4 (Fig. 3C,D). BMP4 was not detected by immunoblotting when the *TSKB* and BMP4 complex was washed under high stringency conditions, although binding of BMP4 to *TSKA* was still detectable (Fig. 3D). Although both *TSKA* and *TSKB* can pull down BMP4 in a dose-dependent manner, the amount of BMP4 precipitated by *TSKA* was higher than that pulled down by *TSKB* (Fig. 3E). The band intensity of lane 2 is three times stronger than that of lane 6, as measured by NIH Image software (data not shown). These data suggest that *TSKA* can bind BMP4 more strongly than *TSKB*. As shown in Fig. 3F, similar to *TSKA*, *TSKB* can also bind to BMP7 (Ohta et al., 2004).

We then examined whether *TSKB* exerts a similar BMP antagonistic activity to *TSKA* (Ohta et al., 2004). It has been shown that the MPS acts as a Hensen's node-inducing center in chick

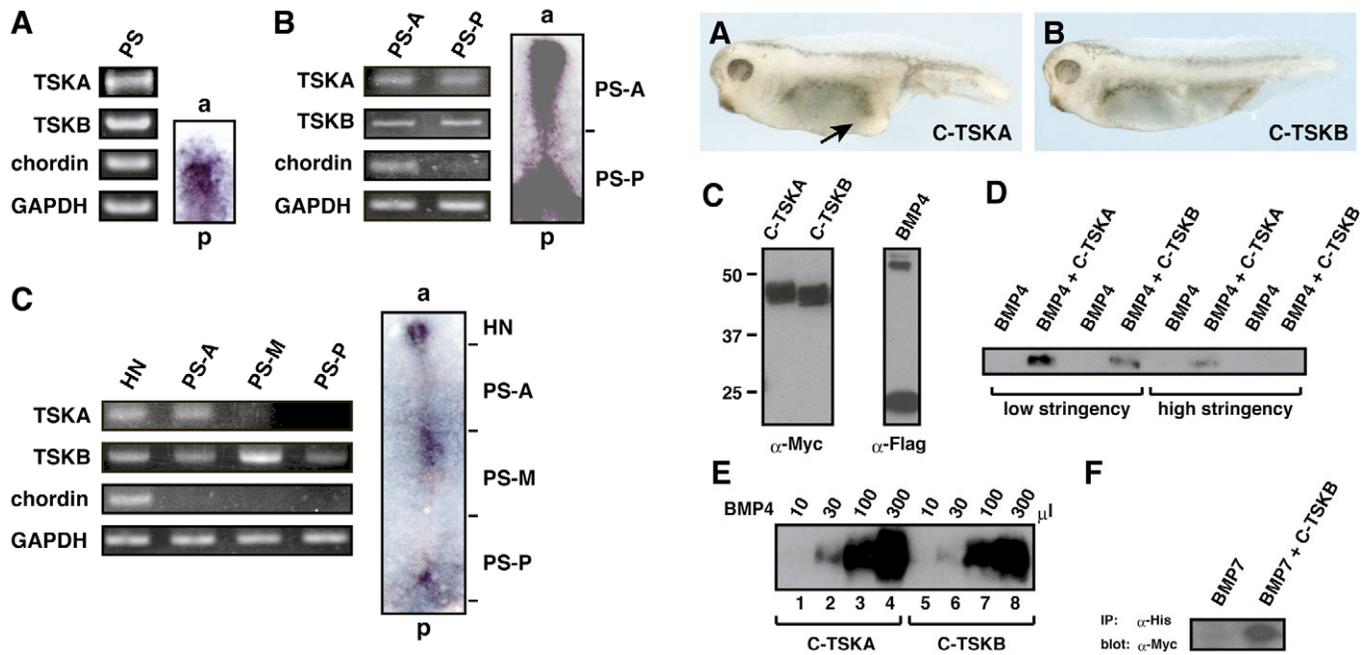


Fig. 2. Expression of *TSKA* and *TSKB* in early chick embryo.

(A–C) In situ hybridization and RT-PCR analyses of chick embryos at stages 2–4. The right panels show the expression patterns of Tsukushi mRNA. (A) Chick embryo at stage 2. (B) Chick embryos at stage 3 were categorized into anterior site (PS-A) and posterior site (PS-P). (C) Chick embryos at stage 4 were categorized into Hensen's node (HN), anterior site (PS-A), middle site (PS-M) and posterior site (PS-P). a, anterior; p, posterior.

embryos, but its activity is inhibited by BMP signals secreted from the periphery of the embryo (Joubin and Stern, 1999). Therefore, aggregates of COS-7 cells expressing *TSKA* or *TSKB* were grafted with the MPS into the lateral edge of a host embryo at stage 3⁺ and cultured for 6 hours. On the other side of the embryo, control COS-7 cells and the MPS were implanted (Fig. 3G). In the presence of *TSKA*, the MPS induced the ectopic expression of chordin in 12/20 (60%) embryos (Fig. 3H), whereas only 3/15 (20%) embryos were positive on the *TSKB* implanted side (Fig. 3I), which was similar to the effect of grafting the MPS together with control cells [6/35 (17%)].

It has also been shown that the node-inducing activity of the MPS depends on and can be mimicked by the expression of both *VG1* and *WNT8C* (Joubin and Stern, 1999). As *WNT1* and *WNT8C* have been shown to belong to the same functional subclass of WNT proteins by several different assays (Joubin and Stern, 1999), we used a stable fibroblast cell line secreting *WNT1* (Shimizu et al., 1997). When *VG1+WNT1* cells were grafted together at the periphery of the area pellucida at stage 3⁺, they showed a weak node-inducing activity [1/8 (13%)] (data not shown). We then grafted *VG1+WNT1* cells along with three aggregates of *TSKA* or *TSKB*-secreting cells (Fig. 3J). In the presence of *TSKA*, *VG1+WNT1* induced ectopic expression of chordin in 6/15 (40%) of embryos (Fig. 3K), while no induction was obtained with *TSKB*-expressing cells [0/16 (0%)] (Fig. 3L), which was similar what obtained by grafting *VG1+WNT1* cells together with control cells [1/31 (3%)]. In conclusion, our experimental results indicate that, although *TSKB* retains some weaker BMP4-binding ability in vitro, its biological activity is much weaker than that of *TSKA*.

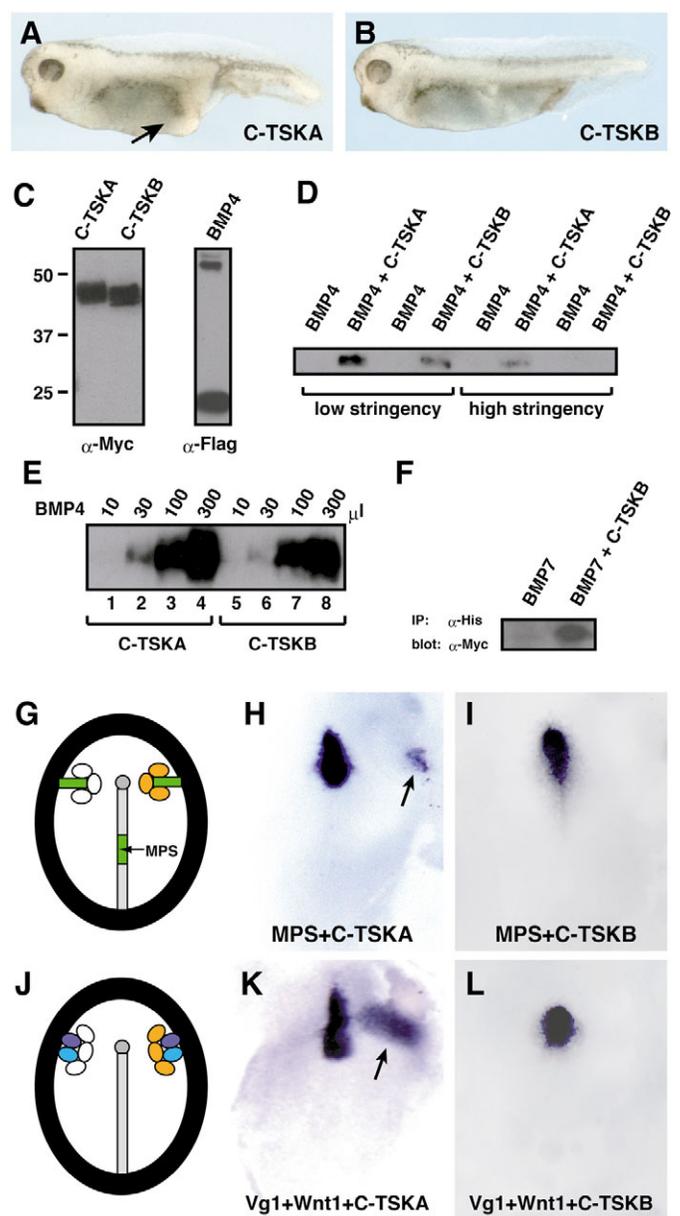


Fig. 3. The different BMP inhibitory activities between *TSKA* and *TSKB*.

TSKA (A) or *TSKB* (B) mRNA was injected into a ventral vegetal blastomere at the eight-cell stage and the embryos were developed until stage 31. Arrow indicates a secondary axis. (C) Western blot analysis of *TSKA*-Myc-His, *TSKB*-Myc-His and *BMP4*-Flag proteins used. (D) Co-immunoprecipitation of *TSKA*-Myc-His or *TSKB*-Myc-His and *BMP4*-Flag. After immunoprecipitation with nickel-chelating resins, the complexes were washed under low or high stringency conditions. (E) Co-immunoprecipitation of *TSKA*-Myc-His or *TSKB*-Myc-His and *BMP4*-Flag with different amounts of *BMP4*. The numbers above the column show the volume (μ l) of COS-7 cell supernatant, including *BMP4* in the total volume (1 ml). (F) Co-immunoprecipitation of *TSKB*-Myc-His and *BMP7*-Flag. (G, J) Transplantation schemes. (H, I) *TSKA*- or *TSKB*-producing cells (orange in G) were grafted together with the middle primitive streak (MPS; green in G) on the right-hand side. The mock-transfected COS-7 cells (white in G) were grafted with MPS on the left-hand side. (K, L) *TSKA* or *TSKB*-producing cells (orange in J) were grafted together with cells expressing *VG1* (purple in J) and *WNT1* (blue in J) on the right-hand side. The mock-transfected COS-7 cells (white) were grafted with cells expressing *VG1* and *WNT1* on the left-hand side.

TSK binds VG1 directly in vitro and functionally interacts with VG1 during axis formation

The PMZ of the chick embryo works as an inducing center responsible for initiating primitive streak formation in the adjacent epiblast (Bachvarova et al., 1998). The molecular synergism between VG1 and WNT activities, whose expression overlaps in the PMZ, is required and sufficient for initiating primitive streak formation (Skromne and Stern, 2001). At later stages, during gastrulation, VG1 and WNT signals are also co-expressed and define a second inducing center in the MPS, where they play a role in the induction of Hensen's node (Joubin and Stern, 1999). The fact that TSKB expression specifically peaks at the MPS (Fig. 2C), suggested to us that TSK also interacts with VG1 in addition to BMPs. To further investigate on this possibility, we compared the expression of TSK, VG1 and WNT8C by at pre-streak stage and during gastrulation. At stage XII, TSK is detected in the area opaca, the PMZ and Koller's sickle (Fig. 4A). WNT8C is expressed all around the area opaca and the marginal zone, most strongly in PMZ, while VG1 is specifically expressed in the PMZ (Fig. 4B,C). At primitive streak stages, both VG1 and WNT8C are expressed in the MPS (Fig. 4E,F) (Joubin and Stern, 1999), as well as TSK (Fig. 4D). Thus, TSK and VG1 expression domains partially overlap both in the PMZ and in the MPS.

We then performed co-immunoprecipitation assays between TSK and VG1. When Myc-His-tagged TSKA or TSKB was reacted with Myc-tagged VG1, immunoprecipitation with nickel-chelating resins specifically pulled down VG1 (Fig. 4G) (Ohta et al., 2004). The binding between both TSKs and VG1 was inhibited by BMP4 (Fig. 4H) (Ohta et al., 2004). As TSK interacts with VG1 in vitro, we subsequently examined whether these molecules can also functionally interact in vivo. To this aim, we initially performed co-overexpression experiments in *Xenopus* embryos. When 100 pg of *VG1* mRNA were injected into a ventral vegetal blastomere of the eight-cell stage embryo, a weak secondary axis was induced lacking any anterior structures [10/21 (47%); Fig. 4I] (Seleiro et al., 1996), while 400 pg of *VG1* mRNA resulted in gastrulation defects (data not shown). However, when 100 pg of *VG1* mRNA were co-injected with 400 pg of *TSKB* mRNA, a well-defined secondary axis was induced in 54% of the cases (26/48). Furthermore, in 17% (8/48) of the embryos a nearly complete secondary axis was induced that also contained anterior structures (Fig. 4J). The TSKA isoform was not suitable for this assay because it can induce a secondary axis by itself (Ohta et al., 2004). These observations suggest that TSK interacts with VG1 during chick early development.

To gain more insight into this interaction, we transplanted TSKA- or TSKB-expressing cells in combination with VG1-producing cells in the marginal zone or in the area pellucida at stage XII and examined the expression of brachyury, a specific marker of the primitive streak mesoderm (Lawson et al., 2001). Fig. 4K shows the schematic diagram of the transplantation experiments. As previously described (Skromne and Stern, 2001), misexpression of VG1 in the area pellucida induced an ectopic primitive streak in only 6/28 (21%) of the grafted embryos, whereas the combination of VG1+WNT1 caused a much stronger effect [9/14 (64%)] (Table 1). When TSKA or TSKB was misexpressed alone in the area pellucida, neither of them could induce ectopic expression of brachyury [TSKA 0/10 (0%), TSKB 0/8 (0%)] (Table 1). By contrast, when TSKA or TSKB were misexpressed in the area pellucida together with VG1, an ectopic primitive streak developed in a significantly higher number of cases compared with VG1 misexpression alone [TSKA+VG1 18/30 (60%), TSKB+VG1 23/46 (50%)] (Fig. 4L, Table 1). Notably, TSK could also strengthen the effect of VG1+WNT misexpression,

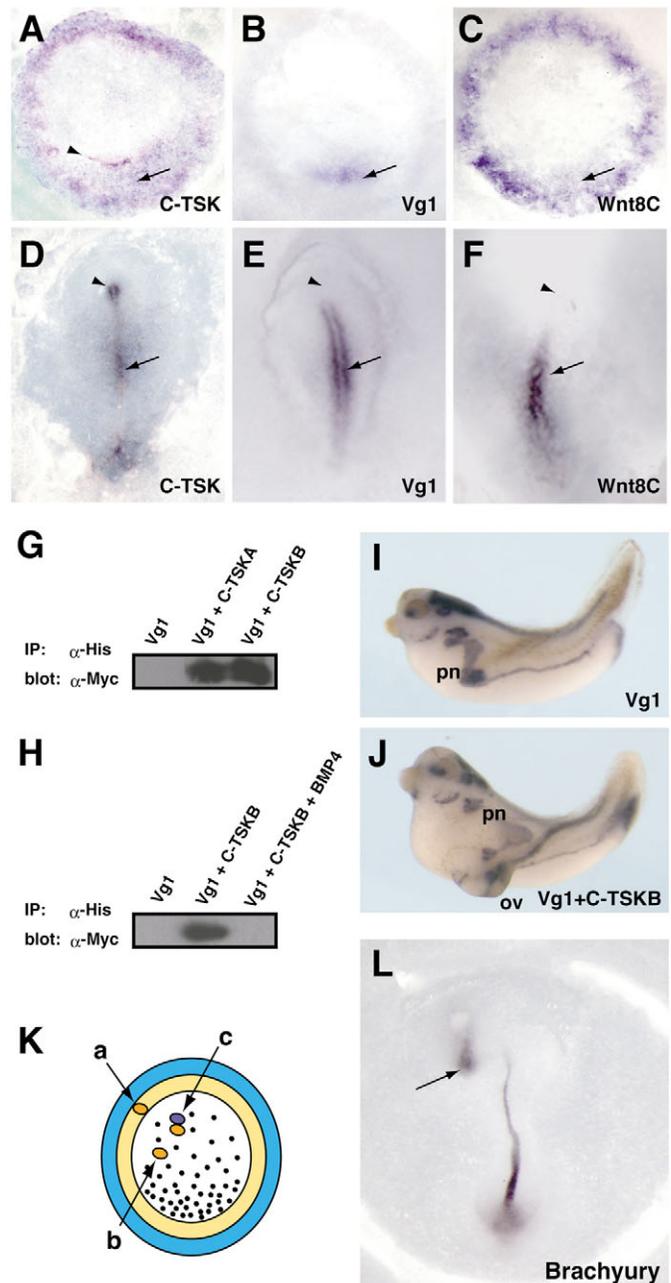


Fig. 4. Tsukushi interacts with VG1. (A-C) Expression of *TSKB* (A), *VG1* (B) and *WNT8C* (C) in stage XII chick embryos. PMZ (arrows); Koller's sickle (arrowhead). (D-F) Expression of *TSKB* (D), *VG1* (E) and *WNT8C* (F) in stage 4 chick embryos. Hensen's node (arrowheads); the middle primitive streak (arrows) (G) Co-immunoprecipitation of TSKA-Myc-His or TSKB-Myc-His and VG1-Myc. After immunoprecipitation with nickel-chelating resins, bound VG1 was detected by immunoblotting with anti-Myc antibody. (H) Co-immunoprecipitation of TSKB-Myc-His and VG1-Myc in the presence of BMP4. (I,J) VG1 mRNA (100 pg) or VG1 mRNA (100 pg) + TSKB mRNA (400 pg) was injected into ventral vegetal blastomeres at the eight-cell stage, and embryos were developed until stage 31 and in situ hybridized with a PAX2 probe. pn, pronephore; ov, optic vesicle. (K) TSKA- or TSKB-producing cells (orange) were grafted into the lateral marginal zone (a), the area opaca (b) or the area pellucida (c) with cells expressing VG1 (purple). (L) Induction of ectopic brachyury (arrow) was observed when TSKB-producing cells were grafted together into the area pellucida ('c' in K) with VG1-expressing cells.

Table 1. Effects of misexpression of TSK and VG1 in the area pellucida

Molecule	Sample/total	%
TSKA	0/8	0
TSKB	0/10	0
VG1	6/28	21
VG1 + WNT1	9/14	64
TSKA + VG1	18/30	60
TSKB + VG1	23/46	50
TSKA + VG1 + WNT1	26/30	87
TSKB + VG1 + WNT1	25/29	86

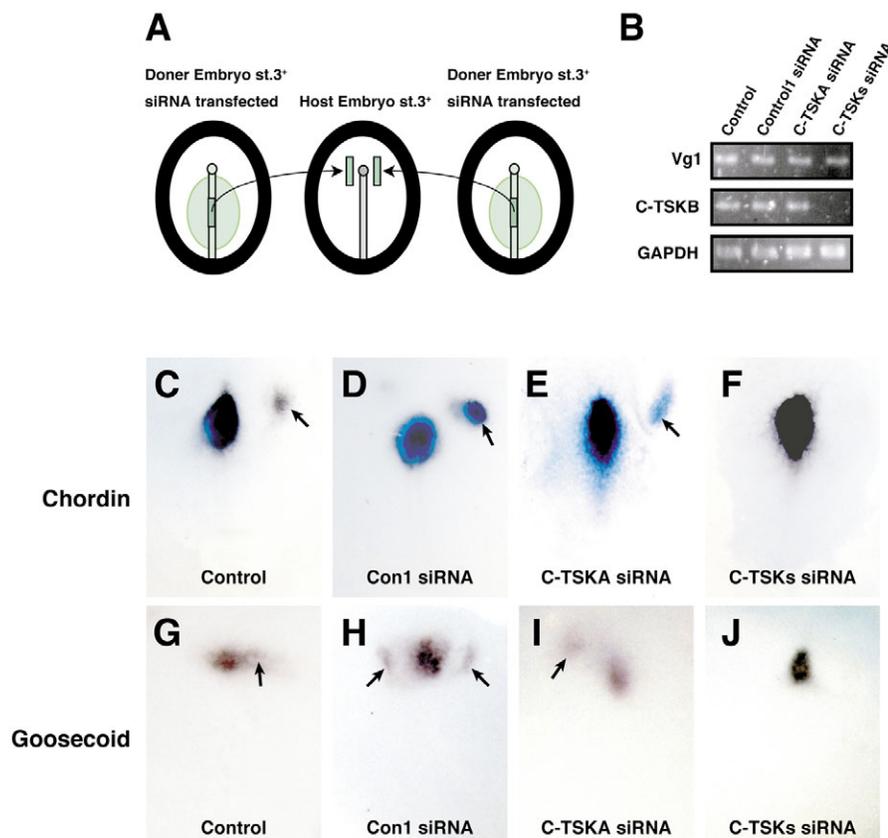
A pellet of cells expressing above molecule was grafted into the area pellucida of pre-streak stage XII embryos; 24 hours later, the ectopic induction of brachyury, a marker for primitive streak, was examined by in situ hybridization.

because, in the presence of TSKA or TSKB, induction of ectopic expression of brachyury raised up to 26/30 (87%) and 25/29 (86%) of embryos, respectively (Table 1). In conclusion, our data suggest that TSK interacts with VG1 during axis formation in the chick embryo.

Loss of TSK function inhibits induction of Hensen's node by the middle primitive streak

The overexpression results suggest that TSK plays an important role in promoting formation of the axial structures by regulating VG1 activity. To confirm this, we performed loss-of-function experiments using siRNAs targeted against TSK mRNA. We employed three different siRNAs: (1) TSKs siRNA, which is targeted against both *TSKA* and *TSKB* isoforms; (2) *TSKA* siRNA, which is specifically targeted against *TSKA*; and (3) control1 siRNA, which does not interfere with TSK expression.

We first verified the effectiveness of these siRNAs by immunoblotting and RT-PCR. COS-7 cells were transfected with control1, *TSKA* or TSKs siRNA, plus plasmid DNA for Myc-His-tagged *TSKA* or TSKB. Immunoblotting of cell culture supernatants showed that *TSKA* and TSKB protein products were nearly fully depleted in the presence of TSKs siRNA, whereas *TSKA* siRNA specifically interfered with *TSKA*, but not TSKB protein expression. *TSKA* and TSKB expression was not affected in the presence of control1 siRNA (see Fig. S1 in the supplementary material). We then checked whether TSKs siRNA is able to downregulate the endogenous expression of TSK. As we have not yet succeeded in electroporating chick embryos at pre-streak stage, we have not been able to use siRNA to interfere with TSK expression in the early-forming primitive streak. As shown in Fig. S2C (see supplementary material), the expression of TSK was even more strongly upregulated in the MPS, which acts to promote node regeneration after surgical removal of the node (Joubin and Stern, 1999). We then analyzed induction of TSK expression after ablation of the node and anterior primitive streak in embryos electroporated with TSKs, *TSKA* or control1 siRNAs. Although TSK expression was upregulated after surgery in control embryos or after electroporation of control1 or *TSKA* siRNA (see Fig. S2C-E in the supplementary material), we hardly detected any expression of TSK mRNA in the MPS of embryos electroporated with TSK siRNA, although some ectopic induction of TSK expression was detectable in both lateral sides of the operated area (see Fig. S2F in the supplementary material). We also examined the effects of TSK depletion on the expression of chordin and goosecoid, which have been used as node markers (Joubin and Stern, 1999), without noticing any evident effects on their expression (see Fig. S2G-N in the supplementary material).

**Fig. 5. Gene silencing of TSK results in the inhibition of organizer formation. (A)**

Transplantation scheme of siRNA experiments. Two chick embryos were electroporated with siRNA at stage 3⁺ and the excised MPSs were implanted into the lateral Hensen's node of the host embryo. **(B)** The embryo at stage 3⁺ was electroporated with control1, *TSKA* or TSKs siRNA. Hensen's node and the anterior fourth of the primitive streak were surgically removed. After 6 hours incubation, the level of *VG1* or *TSKB* mRNA in the MPS was determined by RT-PCR. **(C-J)** After carrying out experiments according to the diagram in A, the ectopic induction of chordin (C-F) or goosecoid (G-J) was examined by in situ hybridization. **(D,E)** In the case of control1 and *TSKA* siRNAs, the MPS induced ectopic expression of chordin in 17/44 (39%) and 12/32 (38%), respectively, which are similar to embryos without electroporation **(C)** [20/50 (40%)]. **(F)** In the case of TSKs siRNA, the MPS induced ectopic expression of chordin in 8/40 (20%) embryos. **(H,I)** In the case of control1 and *TSKA* siRNAs, the MPS induced ectopic expression of goosecoid in 12/42 (29%) and 12/44 (27%), respectively, which are similar to embryos without electroporation **(G)** [12/40 (30%)]. **(J)** In the case of TSKs siRNA, the MPS induced ectopic expression of chordin in 7/53 (13%) embryos.

We showed that *TSKB* RNA accumulates in the MPS during gastrulation (Fig. 2C) and TSK expression is strongly upregulated in the MPS during node regeneration (see Fig. S2C in the supplementary material). As the MPS works as a node-inducing center that specifically expresses VG1 (Joubin and Stern, 1999), we speculated that TSK may play an important role in the MPS by regulating induction of Hensen's node in cooperation with VG1. To test this hypothesis, we carried out an implantation experiments using MPS from either control embryos or from embryos electroporated with TSKs, *TSKA* or control siRNA, and compared their ability to induce an ectopic Hensen's node. Grafts of MPS were implanted into the lateral side of the node into stage 3⁺ host embryos as schematized in Fig. 5A and the expression of organizer markers was analyzed 6 hours later. As previously described (Joubin and Stern, 1999), untransfected primitive streak induced ectopic expression of chordin in 20/50 (40%) embryos (Fig. 5C), which is similar to what was obtained after grafting MPS electroporated with either control1 or *TSKA* siRNA [17/44 (39%) and 12/32 (38%)] embryos with ectopic chordin expression, respectively (Fig. 5D,E). By contrast, transplantation of TSKs siRNA-electroporated MPS resulted in only 8/40 (20%) embryos showing ectopic chordin expression (Fig. 5F). We also used goosecoid as a more specific marker of Hensen's node because chordin is expressed in the notochord and in early organizer precursors as well as in the mature organizer itself (Chapman et al., 2002), and we obtained similar results. In particular, grafts of untransfected, control1 siRNA- and *TSKA* siRNA-electroporated MPS induced ectopic expression of goosecoid in 12/40 (30%), 12/42 (29%) and 12/44 (27%) embryos, respectively (Fig. 5G-I). By contrast, grafts of TSKs siRNA-electroporated MPS induced ectopic goosecoid expression in only 7/53 (13%) embryos (Fig. 5J). Taken together, these results indicate a requirement of TSK activity for Hensen's node induction by the MPS.

TSK acts downstream of VG1 and a VG1-induced inhibitor emitted by the primitive streak

A previous study showed that VG1 induces an unknown secreted inhibitor in the primitive streak that prevents multiple streak formation in chick embryos (Bertocchini et al., 2004). This inhibitor acts downstream of VG1 but upstream of Nodal and Chordin, because VG1+chordin or VG1+Nodal, but not VG1 alone, could efficiently bypass the inhibition (Bertocchini et al., 2004). Does TSK also work downstream of VG1 and this unknown inhibitor? To test this possibility, we first examined whether TSK expression is induced by the misexpression of VG1 in the anterior part of pre-streak embryos. In this assay, VG1 has been shown to induce a cascade of gene activation, including Nodal expression after 6 hours, followed by chordin after 12 hours (Skromme and Stern, 2002). We found that TSK is induced by VG1 after 12 hours (see Fig. S3 in the supplementary material), suggesting that TSK also works downstream of VG1. We then performed further experiments to determine whether TSK works downstream of the VG1-induced inhibitor. We first grafted a pellet of VG1-expressing cells on the lateral margin of stage XII embryos, followed 6 hours later by implantation of another pellet of cells expressing *TSKA*, *TSKB* or other molecules (VG1, chordin, Nodal and FGF8B), which are known to be involved in primitive streak formation (Fig. 6A) (Bertocchini et al., 2004). Weak, if any, ectopic induction of primitive streak took place with any of these molecules (Fig. 6B-D; see Table 2). We then grafted a cell aggregate expressing VG1 on one side, followed 6 hours later by cells expressing VG1 together with one of the above-described molecules on the opposite side (Fig. 6E-H; see Table 2). If TSK works upstream of the VG1-induced inhibitor, co-expression of VG1 and TSK should not be sufficient to induce an ectopic axis. If TSK works downstream of the inhibitor, misexpression of VG1 and TSK could be enough to bypass the inhibitory step. Grafting a second VG1 pellet together with either

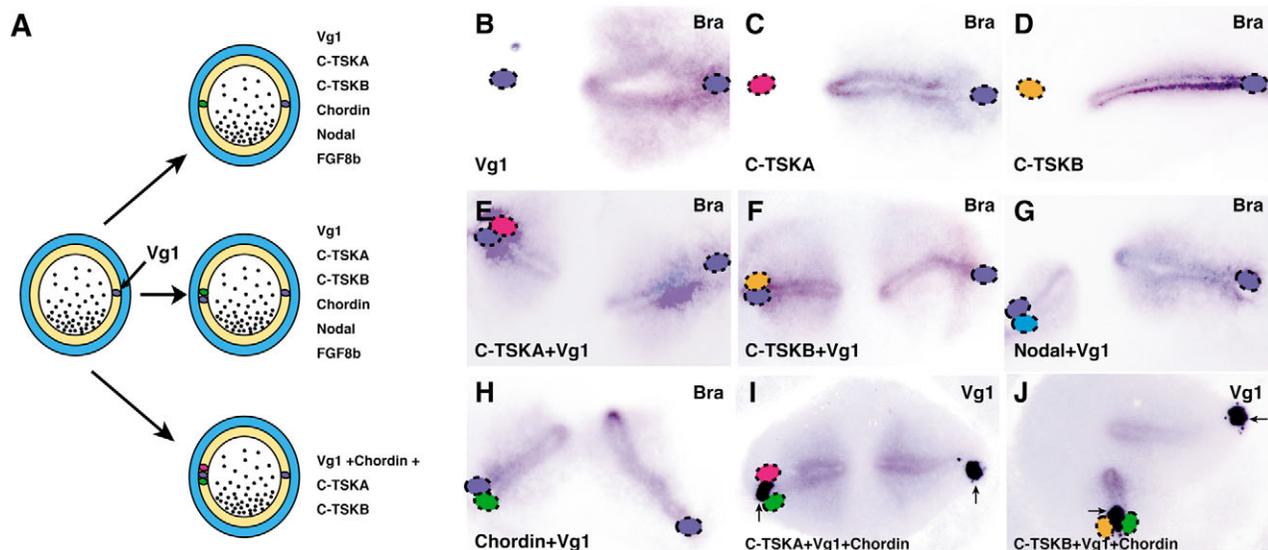


Fig. 6. VG1+TSK can bypass the inhibition by the primitive streak. (A) Grafting a pellet expressing VG1 into the lateral margin of pre-streak stage embryos. Six hours later: (top) a second pellet expressing VG1, TSKA, TSKB, chordin, Nodal or FGF8B; (middle) a combination; (bottom) VG1+chordin + TSKA or TSKB were implanted into the opposite side. (B-D) A pellet of VG1 (B, purple), TSKA (C, pink) or TSKB (D, orange) was implanted into the opposite side. (E-H) A second pellet of VG1 (E-H, purple) and a pellet of TSKA (E, pink), TSKB (F, orange), Nodal (G, blue) or chordin (H, green) were implanted together into the opposite side. (I,J) When VG1 (purple) is implanted laterally, followed 6 hours later by a pellet of cells expressing VG1 (I,J; purple), chordin (I,J, green) and TSKA (I, pink) or TSKB (J, orange) on the opposite side, the combination of VG1+chordin and TSKA or TSKB can induce an ectopic primitive streak. VG1 was used as a probe for in situ hybridization to indicate the cell aggregates expressing VG1 (arrows) in I,J.

Table 2. TSK cooperates with VG1 to induce the primitive streak formation

Molecule	Sample/total	%
TSKA	0/35	0
TSKB	2/38	5
VG1	9/40	23
Chordin	6/54	11
FGF8B	14/66	21
Nodal	1/44	2
TSKA + VG1	21/42	50
TSKA + chordin	7/48	15
TSKA + FGF8B	12/46	26
TSKA + Nodal	0/44	0
TSKB + VG1	37/76	49
TSKB + chordin	6/44	14
TSKB + FGF8B	12/48	25
TSKB + Nodal	2/53	4
VG1 + chordin	22/44	50
VG1 + Nodal	17/43	40
TSKA + VG1 + chordin	39/51	76
TSKB + VG1 + chordin	25/32	78

A pellet of VG1 was grafted into lateral margin of pre-streak stage embryo; 6 hours later, a pellet of cells expressing above molecule was implanted in some combinations.

TSKA, TSKB, Nodal or chordin-expressing cells induced ectopic primitive streak formation [21/42 (50%) embryos with VG1+TSKA; 37/76 (49%) embryos with VG1+TSKB; 17/43 (40%) embryos with VG1+Nodal; 22/44 (50%) embryos with VG1+chordin] (Fig. 6E-H; see Table 2), suggesting that the inhibitor works downstream of VG1 but upstream of TSKs, Nodal and chordin. However, Nodal or chordin alone is not able to induce an ectopic primitive streak in this assay (Bertocchini et al., 2004). Similarly, weak, if any, ectopic induction was obtained by misexpressing chordin or Nodal together with TSKA or TSKB; 7/48 (15%) embryos with chordin+TSKA; 6/44 (14%) embryos with chordin+TSKB; 0/44 (0%) embryos with TSKA+Nodal; 2/53 (4%) embryos with TSKB+Nodal (Table 2). These weak effects are similar to those obtained after transplantation of chordin or Nodal pellets alone [chordin 6/54 (11%); Nodal 1/44 (2%)] (Table 2). Chordin, a strong BMP antagonist (Sasai et al., 1994), has also been shown to act downstream of the VG1-induced inhibitor, since chordin+VG1 could bypass the inhibitory step and induce ectopic primitive streak formation (Bertocchini et al., 2004). However, we found that chordin+VG1 induced an ectopic primitive streak more efficiently when misexpressed together with TSKA or TSKB [39/51 (76%) and 25/32 (78%) embryos, respectively, showing an ectopic primitive streak, compared with 22/44 (50%) embryos] (Fig. 6I,J; see Table 2), suggesting that TSK is not simply working as a BMP antagonist in this assay. Taken together, these results indicate that TSK acts downstream of VG1 and a VG1-induced inhibitor during primitive streak formation.

DISCUSSION

Alternatively spliced variants of the Tsukushi gene

In this paper, we isolated a novel isoform of Tsukushi gene product in chick, *TSKB*, which differs in the structure of the C terminus from *TSKA* (Ohta et al., 2004). Genomic sequence analysis of the TSK gene indicates that these two isoforms are predicted to be generated by alternative 5' splicing of TSK pre-mRNA, a process that is well known to modulate RNA splicing in a developmental and/or cell-type-specific fashion (Cartegni et al., 2002). In the process of alternative 5' splicing, it is believed that cis-acting elements are present in the coding sequences of spliced genes, allowing the

splicing machinery to distinguish between genuine and pseudo-exons and to modulate the selection of alternative splice sites. The mechanisms that are responsible for the recognition and function of these exonic signals are now starting to be elucidated (Cartegni et al., 2002). However, the possible cis-acting elements that may control alternative splicing of the TSK gene are presently unknown. We have identified TSK orthologs in zebrafish, *Xenopus*, mouse and human, all of which have a B-type structure in their C-terminal region (data not shown). Analysis of nucleotide databases did not allow the identification of Tsukushi type A isoforms in these organisms. However, recent progresses in the study of pre-mRNA splicing may open the way to discover type A-related isoforms in other animal species.

TSKA and TSKB isoforms are differentially regulated and possess distinct biochemical activities

Expression analysis of *TSKA* and *TSKB* revealed that they are differentially regulated in chick early development. During initial formation and extension of the primitive streak, both isoforms were co-expressed throughout the developing primitive streak. However, when the primitive streak has reached its full extension, *TSKA* expression is specifically restricted to Hensen's node and the anterior part of primitive streak. By contrast, *TSKB* is expressed throughout the primitive streak, but it is enriched in the MPS. Hensen's node releases BMP-antagonizing signals to pattern the mesoderm and ectoderm tissues (Joubin and Stern, 1999). The MPS instead functions as a node-inducing center because of the specific localization of VG1 and WNT signals (Joubin and Stern, 1999). Therefore, the differential regulation of *TSKA* and *TSKB* expression in Hensen's node and the primitive streak suggests that these TSK isoforms participate in different signaling events during gastrulation.

Indeed, our experimental analysis highlighted a clear difference in the anti-BMP activities of *TSKA* and *TSKB*. We have previously shown that *TSKA* can bind to BMP directly and has a strong anti-BMP activity in vivo (Ohta et al., 2004). This was demonstrated by its ability to induce a secondary axis in *Xenopus* embryos through mesoderm dorsalization and induction of neural tissue, activities that are well known to depend on BMP antagonism in amphibians. In chick embryos, *TSKA* was sufficient and necessary to provide an anti-BMP signal required for Hensen's node induction by the MPS (Ohta et al., 2004; Joubin and Stern, 1999). In contrast to *TSKA*, *TSKB* binds to BMP much more weakly in vitro and shows a much weaker BMP inhibition in vivo (Fig. 3). Taken together, the differential expression patterns and biochemical properties of *TSKA* and *TSKB* suggest that, although *TSKA* is involved in protecting the developing chick axial structures from the inhibitory influence of BMP signals (Ohta et al., 2004), *TSKB* is involved in different signaling activities from BMP antagonism.

TSK isoforms interact with VG1 in the induction of the primitive streak and Hensen's node

The enrichment of *TSKB* expression at the MPS, where VG1 expression is also localized, suggested to us that *TSKB* could interact with VG1 in addition to BMPs. This hypothesis was confirmed by the observation that both *TSKA* and *TSKB* can bind VG1 in vitro. Binding of TSKs to VG1 was competed by BMP4, suggesting that TSKs binds to VG1 and BMPs through at least partially overlapping binding sites. Biological assays performed in *Xenopus* and chick embryos revealed that the interaction between TSKs and VG1 is of a synergistic rather than antagonistic kind.

These results suggested that TSKs functions as an activator of VG1 and modulates the node-inducing activity of the MPS by regulating VG1 function in chick development. We tackled this question directly by loss-of-function experiments using TSK-targeted siRNA (Fig. 5). The fact that TSKA-targeted siRNA was not effective in this assay, and that TSKA RNA expression was hardly detectable in the MPS, suggest that TSKB, but not TSKA, works in the node-inducing center. However, as we were not able to produce an effective TSKB-specific siRNA, this hypothesis could not be formally demonstrated.

VG1 and TSK expression also overlaps in the PMZ at pre-streak stage. The PMZ is known to induce the primitive streak formation, and VG1 and WNT signals mediate this inducing activity at the molecular level (Bachvarova et al., 1998; Skromme and Stern, 2001). Thus, it seems likely that TSK also interacts with VG1 at the level of the PMZ to control the primitive streak formation. This idea is strongly supported by overexpression experiments (Fig. 4). In *Xenopus* embryos, co-overexpression of TSKB together with VG1 caused a much stronger induction of a secondary axis than VG1 alone. In chick embryos, VG1 alone is a poor inducer of primitive streak in the area pellucida, but VG1 and TSKs can induce an ectopic primitive streak efficiently. Thus, the current results support the idea that TSKs regulates VG1 activity and primitive streak induction in the PMZ, although further work will be needed to prove the requirement of TSKs in this process.

The inducing activities of the PMZ and the MPS are both mediated by VG1 and WNT signals (Joubin and Stern, 1999), and the lowered levels of BMP signaling are required in these induction events, because increased BMP levels inhibit both primitive streak and Hensen's node formation (Streit et al., 1998). Could our results be explained with TSK simply working as a BMP antagonist? This seems to be unlikely for the following reasons. First, TSKA and TSKB induce the primitive streak together with VG1 with similar efficiencies. This is in striking contrast to the fact that TSKB is a much weaker BMP antagonist than TSKA. Second, loss-of-function experiments indicate that, in the induction of an ectopic organizer by the MPS, TSKA and TSKB functions are specifically required in Hensen's node and the MPS, respectively [(Ohta et al., 2004), this work, Fig. 5]. The most likely explanation is that induction of an ectopic organizer by the MPS requires two distinct signaling activities: the anti-BMP activity of TSKA secreted from the endogenous node, and a signaling activity of TSKB secreted from the MPS, which is different from BMP antagonism. Finally, when TSKB- or chordin-secreting cells were grafted together with VG1-producing cells on the opposite side of an embryo that was previously grafted with a pellet of VG1-expressing cells into the lateral margin at pre-streak stage, both TSKB and chordin could bypass the inhibitory effects induced by the first VG1 pellet. In this experiment, TSKB could still significantly enhance the primitive streak formation, even when misexpressed in the presence of both VG1 and chordin. In conclusion, our observations indicate that the anti-BMP activity of TSK is not sufficient to explain all of its biological effects, and that an additional signaling activity of TSK, most probably modulation of VG1 function, is involved.

In Fig. 7, we summarize the possible functions of TSK isoforms during chick gastrulation. At pre-streak stage (Fig. 7A), TSK expression is detectable in the area opaca, the PMZ and Koller's sickle. The primitive streak is induced by VG1 and WNT signals secreted from the PMZ, and it requires local lowering of BMP signaling in the posterior epiblast, possibly owing to the action of chordin released from Koller's sickle (Streit et al., 1998). TSK isoforms play a dual role in the process of primitive streak formation.

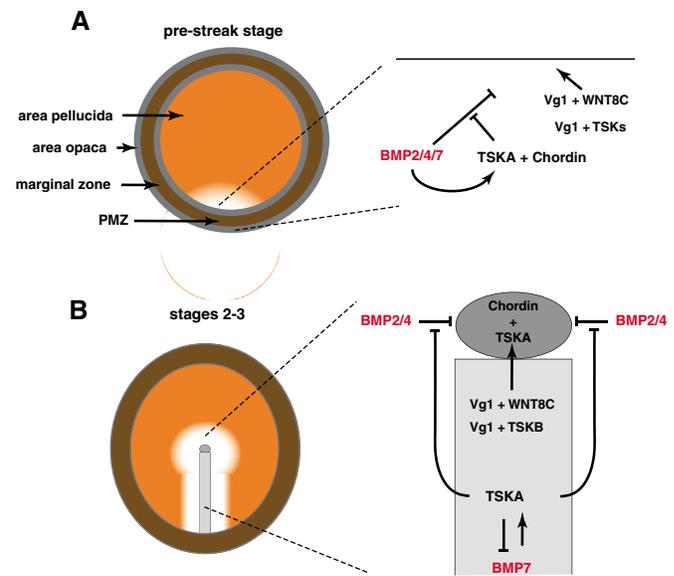


Fig. 7. Model of molecular interactions during primitive streak formation. The successive stages in the development of the chick embryo are shown on the left; the sequential molecular signaling steps are shown on the right. **(A)** Just prior to the gastrulation (stages XI-XIII), WNT8C and TSK are expressed all around the marginal zone, and VG1 in PMZ. TSKA collaborates with chordin to decrease BMP activity in the PMZ. **(B)** At stages 2-3, the primitive streak starts to form under the influence of VG1+WNT8C and VG1+TSKB. VG1+WNT8C and VG1+TSKB signaling cascade synergize to induce the organizer in the central area where the TSKA+chordin serves to keep BMP expression clear of the center. TSKA also inhibits the activity of BMP7 expressed in the posterior primitive streak.

On the one hand, TSKA collaborates with Chordin to decrease BMP activity in the posterior epiblast, in accordance with our previous findings that TSKA and chordin cooperate in blocking BMP signaling (Ohta et al., 2004). Furthermore, the BMP activity positively regulates TSK expression (Kuriyama et al., 2006). On the other hand, both TSKs could positively regulate VG1 activity in the PMZ, thus facilitating induction of the streak. At primitive streak stages (Fig. 7B), both TSKs are initially expressed throughout the primitive streak. However, as the primitive streak reaches its full extension, the expression of TSKA becomes restricted to the anterior part of the streak and Hensen's node, while TSKB expression becomes enriched in the MPS. VG1 and WNT signals secreted from the MPS control the induction of the node at the anterior tip of the streak (Joubin and Stern, 1999). Organizer induction also requires inhibition of BMP2/4 and BMP7 signals, released from the periphery of the epiblast and the posterior streak, respectively (Joubin and Stern, 1999). TSKA is involved in counteracting BMP2/4 signals spreading from the periphery in collaboration with chordin (Ohta et al., 2004), and it may also transiently antagonize BMP7 signals from the posterior streak, thus creating permissive conditions for organizer induction by the MPS. However, TSKA expression is not maintained in the middle streak. By contrast, sustained and increased expression of TSKB in this inductive center may promote node-inducing activity by positively regulating VG1 activity, as highlighted by the present work.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/19/3777/DC1>

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