Development 137, 303-312 (2010) doi:10.1242/dev.040519

FGF signaling directs a center-to-pole expansion of tubulogenesis in mouse testis differentiation

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SUMMARY

In mouse embryogenesis, *Sry* is transiently activated in a center-to-pole wavelike manner along the anteroposterior (AP) axis of developing XY gonads. However, the mechanism and significance of the center-to-pole expansion of testis initiation pathways downstream of *Sry* expression remain unclear. Here we demonstrate that FGF9 can act as a diffusible conductor for a poleward expansion of tubulogenic programs at early phases of testis differentiation. In XY genital ridge cultures of anterior, middle and posterior segments at 11.0-11.25 days post-coitum, male-specific activation of *Sry* and its target gene, *Sox9*, was still observed in both anterior and posterior pole segments despite their isolation from the central domain. However, high-level *Sox9* expression was not maintained, resulting in the failure of testis cord organization in most pole segments. A reconstruction experiment using ROSA:*lacZ* middle segments showed rescue of the tubulogenic defect in the poles without any appreciable contribution of *lacZ*-positive gonadal parenchyma cells. A partition culture assay also showed a possible contribution of soluble/diffusible factors secreted from the gonadal center domain to proper tubulogenesis in the poles. Among various signaling factors, *Fgf9* expression was significantly lower in both anterior and posterior pole segments than in the central domain. The supportive role of the central domain could be substituted by exogenous FGF9 supply, whereas reduction of *Wnt4* activity did not rescue the tubulogenesis defect in the pole segments. These observations imply that center-to-pole FGF9 diffusion directs a poleward expansion of testiculogenic programs along the AP axis of developing XY gonads.

KEY WORDS: *Sry*, *Sox9*, *Fgf9*, Sertoli cell, Sex differentiation, Gonad, Anteroposterior axis, Mouse

INTRODUCTION

In mammals, *Sry* (sex determining region of Chr Y) is cell-autonomously essential in the supporting cells for initiating Sertoli cell differentiation and testis formation (Sinclair et al., 1990; Gubbay et al., 1990; Koopman et al., 1991). In mice, *Sry* expression is first detected in the central region of the XY gonad at 11.0 days post-coitum (dpc) – approximately at the 12 tail-somite (ts) stage – with its expression extending to both the anterior and posterior ends by 11.5 dpc (approximately 18 ts) (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001). Thereafter, *Sry* expression is rapidly reduced in the central domain and becomes restricted to the posterior pole before completely disappearing at 12.5 dpc (approximately 30 ts). Shortly after the onset of *Sry* expression (i.e. approximately 14-15 ts), various testis-specific genes including *Sry*-target genes, *Sox9* and *Cbln4*, are upregulated in a center-to-pole manner similar to that of the initial *Sry* expression profile (Moreno-Mendoza et al., 2003; Schepers et al., 2003; Bradford et al., 2009). A similar center-to-pole pattern is also found in the potency to induce testis cord formation in the cultures of anterior, middle and posterior pole segments of the XY genital ridge (Hiramatsu et al., 2003). However, the biological significance of the poleward expansion in testiculogenic programs also remains unclear because no structural and functional distinctions along the anteroposterior (AP) axis are seen in either testes or ovaries at later stages. Moreover, it is still unclear how spatiotemporal patterns of *Sry*-downstream testis-determination pathways are coordinately regulated in the gonadal parenchyma cells aligned along the AP axis.

Recently, we demonstrated that forced ubiquitous expression of transgene-derived *Sry* transcripts in the entire gonadal area from stages earlier than 9 ts results in neither advance in timing nor ectopic activation of *Sox9* expression in developing gonads (Kidokoro et al., 2005). This suggests that *Sry* alone cannot drive center-to-pole expansion of testiculogenic programs at the initial phase of testis differentiation. Moreover, we previously found, using a novel *Sry*-inducible transgenic system, that *Sry* is able to ensure testis formation in XX gonads only if it is expressed during the period of approximately 11.0-11.25 dpc (i.e. 12-14 ts), which corresponds to a time window of only 6 hours after the normal onset of *Sry* expression in XY gonads (Hiramatsu et al., 2009). These findings suggest that the initial 6 hour phase of *Sry* expression is crucial for eventual testicular development along the whole length of the gonad, despite the fact that, endogenously, *Sry* expression is observed in the central region only during this initial 6 hour period. Moreover, in our previous study, in the segment assay using XY genital ridge cultures of anterior, middle and posterior segments at this initial 6 hour window, the removal of the central domain clearly caused defective formation of testis cord in both anterior and posterior pole segments (Hiramatsu et al., 2003). Hence, we hypothesised that the *Sry* action in the central domain during the 6 hour window is to trigger production of a factor that acts non-cell-autonomously to influence the adoption of testiculogenesis in the anterior and posterior poles.

In this study, by using the segment, reconstruction and partition culture assays, we provide evidence showing the supportive role of the center-derived FGF9 signals in the proper progression of testis formation in the anterior and posterior poles of developing XY gonads.

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Accepted 10 November 2009

MATERIALS AND METHODS

Animals

Embryos were obtained from pregnant female mice (ICR strain) from 11.0-11.5 dpc (12-18 ts). In some experiments, the XX/*Sry* sex-reversal transgenic line [#40 (Kidokoro et al., 2005); ICR/C57BL6[B6]-mixed background], ROSA26 line (ICR/B6-mixed background) and *Wnt4*-heterozygote line (129svJ/ICR-mixed background) were also used. After counting the ts number, genital ridges were used for the experiments described below. The sex and genotype of each embryo was determined by PCR as described previously (Kidokoro et al., 2005; Mizusaki et al., 2003).

Organ culture

A segment culture assay of XY genital ridges at 12-14 ts stages (i.e. 11.0–11.25 dpc) was performed as described previously (Hiramatsu et al., 2003). One of each pair of genital ridges was separated into three equal segments (i.e. anterior, middle and posterior) using a sharp needle under a dissecting microscope. The other was used as a whole gonadal explant for the control experiment.

In the segment and reconstruction assay, XY genital ridges were isolated from ROSA26 (*lacZ*+) and wild-type embryos at 12-14 ts (see Fig. 3A,B). After genotyping, the XY genital ridges were separated into three equal segments, and then the wild-type:anterior, ROSA26:middle and wild-type:posterior segments were assembled in order and recombined on 1.5% agarose blocks by 12 hour incubation with DMEM (Sigma-Aldrich) containing 10% horse serum (GIBCO-BRL).

In the partition culture assay, the gonadal or mesonephric region of the isolated XY genital ridge was partitioned into two (anterior and middle-posterior) segments using aluminum foil sheet or 3 μm Nuclepore filter in Mebiol Gel (thermoreversible polymer; Mebiol, Japan) (see Fig. 4A). All genital ridge explants were cultured in 24-well tissue culture plates with 10% horse serum-DMEM at 37°C for appropriate periods (3 hours to 5 days). The culture medium was changed every 24 hours. Some segment explants were cultured with 10% horse serum-DMEM containing FGF9 (50 ng/ml; R&D systems), IGF1 (50 ng/ml; Sigma-Aldrich), PDGF-BB (50 ng/ml; Sigma-Aldrich), HGF (100 ng/ml; Nacalai), NGF (100 ng/ml; kindly provided by Drs Stainsz and Bienenstock, McMaster University) or PGD2 (500 nM; Cayman Chemical) for 24 hours and then further cultured in the control medium (without additive) for 4 days. Some whole genital ridge explants were also cultured in medium containing the FGF inhibitor SU5402 (10 μM; Calbiochem) for 12-16 hours. In addition, we performed at least three separate experiments for each culture assay.

lacZ staining

The reconstructed explants were fixed in 0.2% glutaraldehyde-1% paraformaldehyde (PFA)-0.02% NP40-PBS, and subjected to whole-mount X-gal staining (Kanai-Azuma et al., 2002).

Histology, lectin histochemistry and immunohistochemistry

For histological analysis, the explants were fixed in 2.5% glutaraldehyde-0.1 M phosphate buffer (PB) and embedded in Araldite-M (Nisshin-EM, Japan). Semithin sections (approximately 1 μm) were prepared and stained with 1% Toluidine Blue.

For lectin histochemistry and immunohistochemistry, the explants were fixed in 4% PFA and routinely embedded in paraffin. Serial sections (4 μm) were incubated with anti-SRY antibody [1/50 dilution (Wilhelm et al., 2005); kindly provided by Drs Wilhelm and Koopman, The University of Queensland], anti-laminin antibody (1/400 dilution; ICN Pharmaceuticals), anti-SOX9 antibody (10 μg/ml) (Kidokoro et al., 2005), or biotin-labeled endothelial cell-specific isolectin GS-I (25 μg/ml; Vector Laboratories) (Porter et al., 1990). After incubation with biotin-labeled secondary antibodies for immunostaining, the immunoreaction and lectin binding were visualized using an ABC kit (Vector Laboratories).

The presence of testicular cord formation was estimated using the Toluidine Blue stained semithin and anti-Laminin stained sections as follows: negative (–), no cord-like structure; ±, slender cord-like structure; positive (+), well-defined testicular cords in the gonadal area (Hiramatsu et al., 2003). For quantitative analysis of the number of SOX9-positive cells, the number of cells was counted in three sagittal sections per explant (Kidokoro et al., 2005). The cell number per area (cell number per 10,000 μm^2) was calculated separately in each segment.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using 4% PFA-fixed explants as described previously (Hiramatsu et al., 2003). Hybridization was carried out for 16 hours at 68°C for *Sry* (Bullejos and Koopman, 2001), *Sox9* (Kent et al., 1996), *Dusp6* (Li et al., 2007) and *Wnt4* (Mizusaki et al., 2003), or at 55°C for *Fgf9* (Colvin et al., 1999) and *Fgf10* (Harada et al., 2002).

Quantitative RT-PCR

Total RNA was extracted from the gonadal area of the explants using Trizol reagent (Invitrogen). Each RNA sample was reverse transcribed using random primer with a Superscript-III cDNA Synthesis Kit (Invitrogen). A reverse transcriptase-free reaction was performed as a control. Specific primers and fluorogenic probes for *Fgf9* (Mm00442795_m1), *Fgf10* (Mm01297079_m1), *Igf1* (Mm00439560_m1), *Pdgfb* (Mm01298578_m1), *Pgds* (Mm01330613_m1), *Hgf* (Mm01135185_m1), *Wnt4* (Mm00437341_m1) and *Gapdh* (Taqman control reagents) were purchased from Applied Biosystems. PCR was performed using an ABI Prism 7900HT sequence detector. The expression levels represented the relative expression levels of each marker gene per *Gapdh* amplicon ratio (mean ± standard error).

Statistical analysis

Quantitative expression data by real-time PCR analyses and relative cell number of SOX9-positive cells were analyzed using Student's *t*-test. Tubulogenic capacities in segment explants were analyzed using the χ^2 test.

RESULTS

Segmentation into three equal pieces along the AP axis does not affect center-to-pole *Sry* expression in XY genital ridge explants initiated at 12-14 ts

Our previous segment assay of 5 day genital ridge cultures demonstrated that spatiotemporal changes in testiculogenic capacity occur dynamically at the early phase of testis formation (Hiramatsu et al., 2003). It was shown that the capacity to induce testis cord formation extends in a center-to-pole manner after 15 ts (11.3 dpc), which coincides with the time when *Sry* expression reaches the poles in vivo. First, in order to clarify the contribution of *Sry* expression to defective cord formation in the anterior/posterior pole domains, we examined the temporal expression profiles of endogenous *Sry* gene in anterior, middle and posterior segments of XY genital ridges (ICR strain) at 12 ts (Fig. 1A,B). In whole explants using intact XY genital ridges at 12 ts, *Sry* expression was detectable only in the middle region at 3 hours, and its expression domain expanded to both anterior and posterior ends after incubation for 9 hours (left in Fig. 1A). In segment cultures of XY genital ridges at the same stage (12 ts), *Sry* expression was seen only in the middle segments at 3 hours. After 9 hours, its expression was detected in all three segments, showing a similar center-to-pole pattern as in the intact whole explants. Anti-SRY immunohistochemical analysis also confirmed that SRY proteins were properly expressed in the nucleus of pre-Sertoli cells, not only in the middle segment but also in the anterior and posterior segments of XY genital ridges at 12-13 ts (Fig. 1B). These findings indicate that SRY expression properly occurs in the anterior and posterior segment explants initiated at 12-14 ts.

In order to examine the influence of forced *Sry* expression on the tubulogenic capacity of the anterior and posterior pole domains, we also performed a segment culture assay using the XX sex-reversal transgenic (Tg) line (XX/*Sry*; ICR/B6 mixed background) carrying the *Hsp-Sry* transgene (Kidokoro et al., 2005). In this sex reversal line, the transgene-derived *Sry* transcripts are ubiquitously expressed throughout the entire gonadal area of the XX/*Sry* genital ridge at

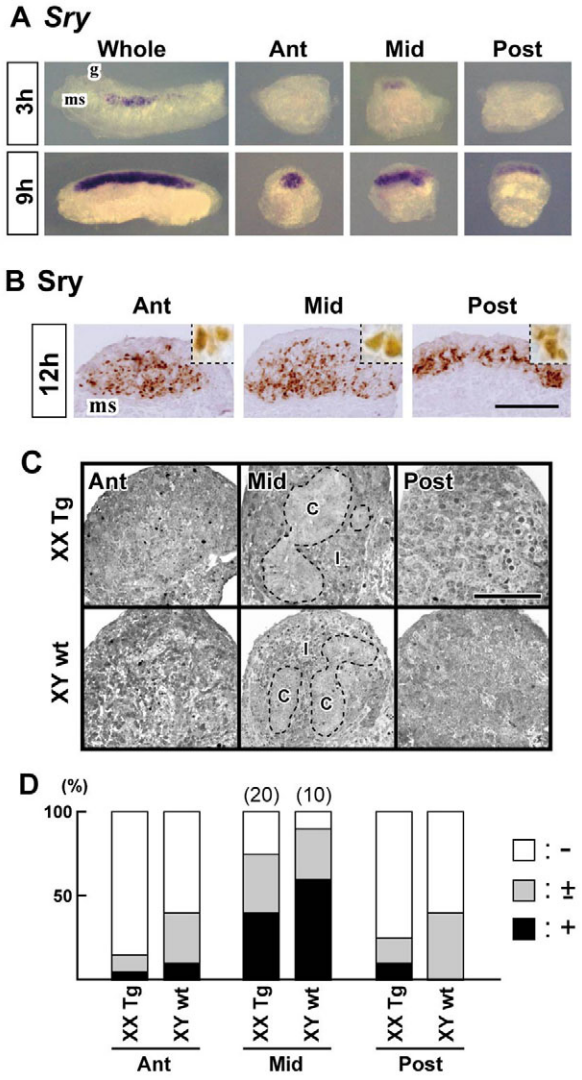

Fig. 1. Proper SRY expression, but defective tubulogenesis, in the pole segment explants of XY genital ridges. (**A**,**B**) Whole-mount in situ hybridization (A) and immunohistochemical (B) analyses showing *Sry* expression patterns in anterior (Ant), middle (Mid) and posterior (Post) segment explants of XY genital ridges (initiated at 12 ts) after incubation for 3, 9 and 12 hours. In A, left panels show *Sry* expression in the control explants of the whole genital ridge of the same embryo. In B, anti-SRY staining showing that SRY proteins are properly expressed in the nucleus of the pre-Sertoli cells (insets). (**C**,**D**) Histological analysis of semithin sections showing defective testiculogenesis in anterior/posterior segments of the XY genital ridges (12-14 ts; 5 days culture) of XX/*Sry* transgenic embryos (ubiquitous SRY expression in entire gonadal area from 9 ts) (Kidokoro et al., 2005) and XY wild-type littermates. In D, percentile graphs show relative number of each segment explant with no cord-like structure (white, –), slender cord-like structure (gray, ±) or well-defined testicular cords (black, +). The number in parentheses indicates the number of explants used to estimate the cord formation. C, testis cord; g, gonad; I, interstitum; ms, mesonephros. Scale bar: 100 μm.

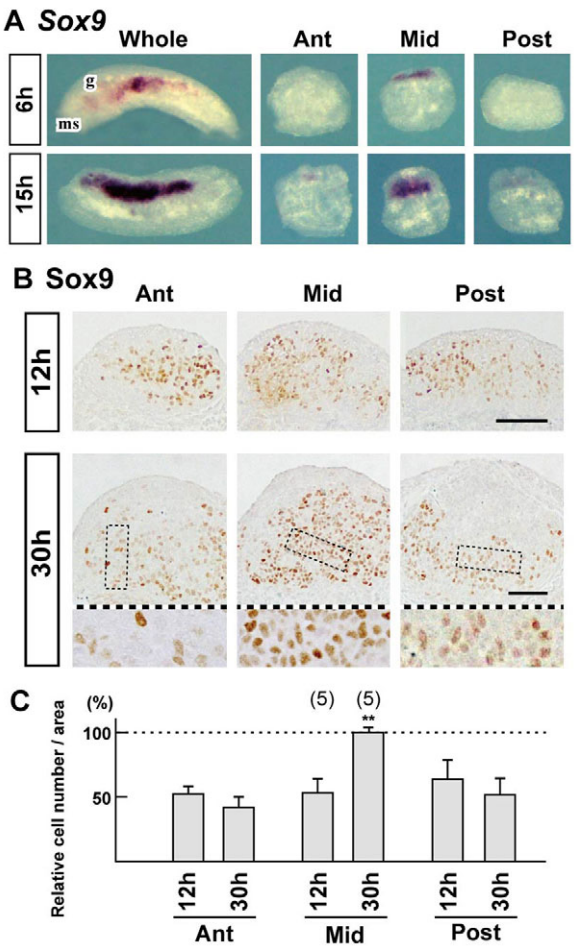

Fig. 2. Defective maintenance of high-level *Sox9* expression and reduced number of SOX9-positive cells in the pole segment explants. (**A**) In situ hybridization analysis showing *Sox9* expression patterns in the control explants of the whole genital ridge and in the segmented explants of the same XY embryo (12 ts) after incubation for 6 and 15 hours. (**B**,**C**) Anti-SOX9 immunostaining showing the proper initiation of SOX9 expression, but no appreciable expansion in either cell number or staining intensity in the anterior/posterior segments at 30 hours. In B, insets show higher magnified images indicated by dashed rectangles in upper panels. In C, bar graphs show the relative cell number of SOX9-positive cells per gonadal area in the segment explants (initiated at 12 ts) after incubation for 12 and 30 hours (the mean values of the cell number ± standard error; n=5). The value in the middle segment explants at 30 hours is set as 100% ($19.6\pm0.6\times10^3$ cells per mm^2). The value in the middle segments at 30 hours is significantly (**P<0.01) higher than all other values in segment explants. g, gonad; ms, mesonephros. Scale bar: 100 μm.

least from 9 ts (approximately 10.5 dpc). We isolated three segments of the genital ridges from XX/*Sry* Tg and XY wild-type embryos at 12-13 ts, and estimated their capacities to induce cord formation in 5 day cultures (Fig. 1C,D). Despite ubiquitous SRY expression in anterior/posterior domains, the tubulogenic capacity was not significantly improved in the anterior/posterior segments of XX/*Sry* genital ridges, compared with those in the wild-type XY explants. In addition to the tubulogenic potency being significantly lower in both the anterior and posterior segments than that in the middle segment of XX/*Sry* Tg genital ridges, no testis cord formation was observed in any anterior/posterior segments even in XY/*Sry* Tg genital ridges (n=3). Therefore, these data indicate that defective cord formation in the pole segments within the initial 6 hour phase is not directly caused by deficiency of SRY expression itself.

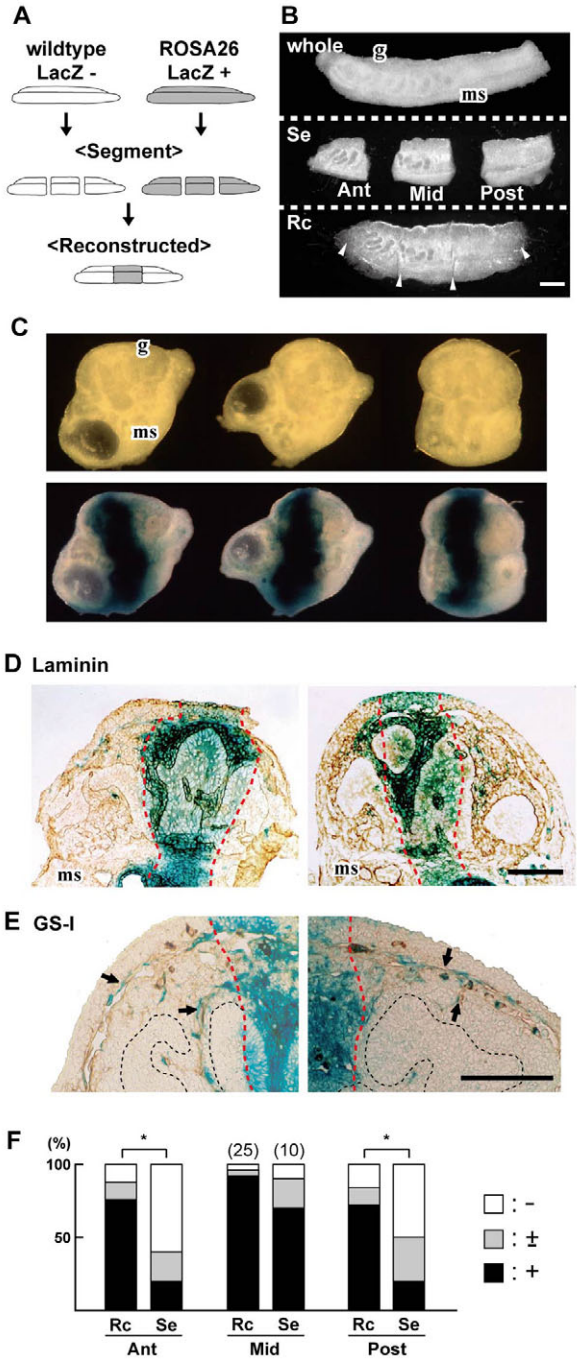

Fig. 3. A segment and reconstruction culture assay of the anterior and posterior pole segments with the middle segment of XY genital ridge. (**A**,**B**) A schematic representation showing the reconstruction of the ROSA26:*lacZ* middle and wild-type anterior/posterior segments of the XY genital ridges isolated at 12-14 ts. In B, phase-contrast images show a whole genital ridge at 13 ts ('whole'), three equally sized segments ('Se') and their reconstructed explant (1 hour culture; white arrowheads, cutting planes) ('Rc'). (**C**) Phase-contrast images showing three reconstructed explants (5 days culture). Lower panel shows whole-mount *lacZ* staining images of three explants shown in the upper panel. (**D**,**E**) Anti-laminin immunostaining (D) and GS-I histochemical staining (E) of the *lacZ*-stained reconstructed explants. In D and E, broken red lines indicate the well-defined boundary lines between the middle and pole segments, whereas broken black lines in E show the boundary of the testis cords. In E, arrows show a small population of *lacZ*-positive cells in the poles that are closely associated with GS-I-positive endothelial cells in the interstitum. (**F**) Percentile graphs show relative number of the segment and recontruction explants with no cord-like structure (white), slender cord-like structure (gray) or well-defined testicular cords (black) in their anterior, middle and posterior regions. Statistical analysis (Fisher's exact test) shows significant recovery in the tubulogenesis of the pole regions in reconstructed explants (*P<0.05). g, gonad; ms, mesonephros. Scale bar: 100 μm.

Defective maintenance of high-level *Sox9* expression in anterior/posterior pole domains in segment cultures of XY genital ridges

Next, we examined the timecourse expression pattern of *Sox9,* an *Sry*-target gene (Sekido and Lovell-Badge, 2008), in segment explants of XY genital ridges at 12 ts by whole-mount in situ hybridization. In whole explants of intact XY genital ridges, *Sox9* was upregulated in the middle domain at 6 hours, and its expression domain expanded to the anterior/posterior poles after incubation for 12 hours (left in Fig. 2A). In the middle segments of XY genital ridges at 12 ts, *Sox9* expression was observed at 6 hours, and its expression appeared to be increased after incubation for 12 hours. By contrast, in most anterior and posterior segments, *Sox9* expression was detected at only a faint level even at 15 hours after culture initiation (Fig. 2A).

Interestingly, anti-SOX9 immunochemical analysis revealed that SOX9-postive cells are detectable in both anterior and posterior segments, showing a similar expression pattern to that in the middle segments after incubation for 12 hours (upper panels in Fig. 2B). The differences between these RNA and protein data are possibly due to the higher detection sensitivity of anti-SOX9 immunostaining than that of whole-mount in situ hybridization in the present experimental conditions. After incubation for 30 hours, we observed an increase in both cell number and staining intensity of SOX9-positive cells in the middle segments (Fig. 2B,C). In the anterior and posterior segments, a small population of high SOX9-positive cells was also observed in the gonadal area, but no significant increase was detected in either cell number or signal intensity compared with those in the middle segments. These results suggest that *Sox9* expression is properly, albeit weakly, initiated in pre-Sertoli cells in the anterior and posterior segments, but the maintenance of its expression, and/or recruitment of precursor cells, is defective in the pole segments. It is likely that this defective maintenance of pre-Sertoli cells leads to the failure of testis cord formation in anterior and posterior pole segments of XY genital ridges in vitro. In addition, no appreciable differences are found in apoptotic cell death among the anterior, middle and posterior segment explants (see Fig. S1 in the supplementary material).

Both reconstruction and partition experiments show a possible importance of the contribution of certain soluble/diffusible factors secreted from the middle segment to proper testiculogenesis in the pole domains

This study suggests that the removal of the central domain represses the capacity of the anterior/posterior domains to maintain high SOX9-positive cells and subsequent testis cord formation in XY genital ridge explants initiated at 12-14 ts. Next, in order to clarify the contribution of the middle segment to the anterior and posterior regions, we performed a reconstruction assay using ROSA26:*lacZ* middle and wild-type anterior/posterior pole segments of XY genital ridges at 12-14 ts (Fig. 3A,B). In brief, we recombined ROSA26:*lacZ* middle and wild-type anterior/posterior segments to create one whole genital ridge, and then cultured them for 4 days.

Under a phase contrast microscope, most of the reconstructed whole genital ridges properly developed into one explant showing gross anatomical similarity to that of intact whole explants (upper panel in Fig. 3C). *lacZ* staining of the reconstructed explants revealed that well-defined borders were formed between middle and pole segments, indicating low levels of cell movement along the AP axis of developing XY genital ridges (Fig. 3C,D). Histological analysis revealed that testis cord formation was properly induced in the *lacZ*-negative anterior/posterior regions in approximately 70% of the reconstructed explants (Fig. 3D,E; 'Rc' in Fig. 3F). This finding is in sharp contrast with the observation that testis cord formation occurs only in approximately 20% of anterior and posterior segment explants ('Se' in Fig. 3F). These results confirm that the presence of the middle segment is able to rescue defective testis cord formation in the anterior/posterior segments at this stage.

Anti-laminin staining also revealed that *lacZ*-positive and -negative Sertoli cells combined to organize into the testis cord structures in the border areas between the middle and pole segments (broken red lines in Fig. 3D,E). Although a small population of *lacZ*-positive cells was seen in the anterior and posterior pole regions (*lacZ*-negative), lectin staining of GS-I, a marker for capillary endothelial cells (Porter et al., 1990), showed that most *lacZ*-positive cells in the pole regions were closely associated with the developing blood vessels in the interstitial and subepithelial regions (arrows in Fig. 3E), suggesting testis-specific vasculogenesis (Brennan et al., 2002). These data suggest that the middle segment promotes testis cord formation in the anterior/posterior segments without any appreciable contribution of gonadal parenchyma cells. This in turn highlights a possible contribution of soluble/diffusible factors secreted from the middle domain.

To confirm the contribution of center-derived soluble factors, we also partitioned the gonadal or mesonephric region of the whole genital ridge (at 12-13 ts) into two (i.e. anterior and middle-posterior) domains using an aluminum foil sheet (a complete barrier) or 3 μm Nuclepore filter (a barrier to cell migration, not to soluble factors), and then cultured them for 4 days (Fig. 4A,B). In the XY genital ridge explants with the mesonephros partitioned by aluminum foil, it was shown that the testis cord formation was properly induced in the anterior domain in most of them ('ms-cut' in Fig. 4C,D). By contrast, the partition in the gonadal region by the aluminum foil significantly repressed the cord formation in the anterior domain ('gd-cut' in Fig. 4C,D), suggesting a possible contribution of gonadal region, rather than mesonephric region, to testiculogenesis in the anterior domain. Moreover, the partition by the 3 μm Nuclepore filter showed the rescue of defective testis cord formation in the anterior domain ('gd-filter' in Fig. 4C,D). Therefore, these data strongly indicate the importance of the contribution of soluble/diffusible factors secreted from the gonadal center domain (at the advanced stage of testis initiation) to proper testiculogenesis in the pole domains of developing XY gonads.

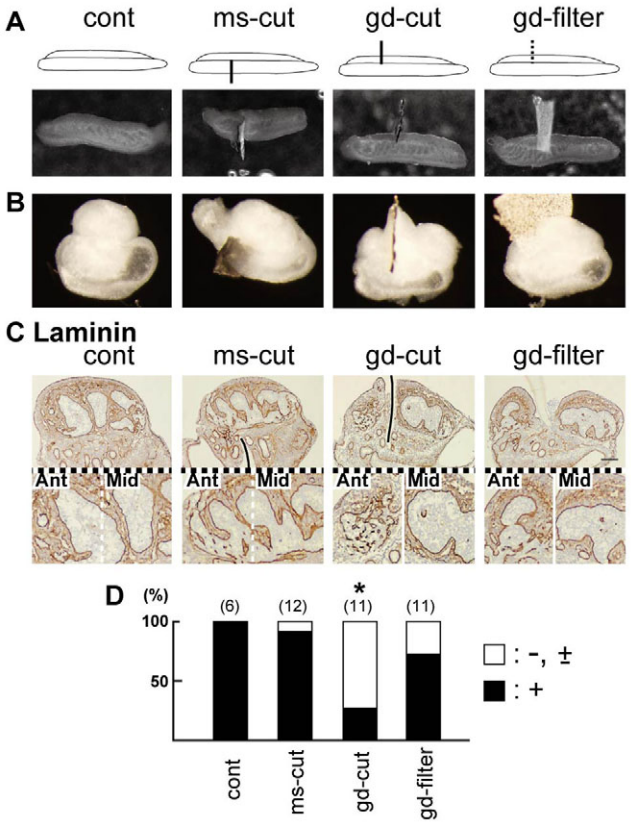

Fig. 4. A partition culture assay of whole XY genital ridge explants. (**A**,**B**) A schematic representation (upper panels in A) and phase-contrast images (lower plates in A; B) of the partition culture assay showing the whole XY genital ridges (initiated at 12-13 ts, 1 hour (A) or 4 day (B) culture) partially divided by the barrier [aluminum foil sheet (cut) or 3 μm Nuclepore filter (filter)] in the anterior one-third of the gonadal (gd-cut, gd-filter) or mesonephric (ms-cut) site. (**C**,**D**) Anti-laminin immunostaining of the partition explants, showing defective cord formation in the anterior region of the 'gd-cut' explant, but proper tubulogenesis in those of the 'ms-cut' and 'gd-filter' explants. In C, lower plates show higher magnified images of anterior and middle gonadal regions. In D, percentile graphs show relative number of the explants with no cord-like or disorganized cord-like structure (white) or well-defined testicular cords (black) in the anterior pole regions. Statistical analysis (Fisher's exact test) shows a significantly lower level (*P<0.05) in the tubulogenesis of the anterior region in the 'gd-cut' explants, compared with those in the other explants. Scale bar: 100 μm.

FGF9 is able to mimic the supportive roles of the middle segment to induce testis cord formation in anterior and posterior pole segments

It has previously been shown that FGF9 signaling is crucial for the maintenance of *Sox9* expression and testis cord formation in developing XY gonads (Colvin et al., 2001; Kim et al., 2006). Moreover, various other signaling molecules, such as PDGFB (platelet derived growth factor B) (Brennan et al., 2003; Puglianiello et al., 2004; Ricci et al., 2004), PGD2 [prostaglandin D2 (PTGDS – Mouse Genome Informatics)] (Malki et al., 2005; Wilhelm et al., 2005; Wilhelm et al., 2007), neurotrophins (Cupp et al., 2000), HGF (Ricci et al., 1999) and insulin/IGF (Nef et al., 2003), have been shown to be involved in testiculogenesis in developing mouse embryos. First, we quantitatively examined the mRNA expression levels of the center-derived candidate factors (*Fgf9*, *Igf1*, *Pdgfb*, *Pgds* and *Hgf*) in the anterior, middle and posterior explants of the XY genital ridges initiated at 12-13 ts (18 hours culture). Among them, the expression levels of *Fgf9* and *Pgds* were significantly lower in both anterior and posterior segments than those in the middle segments (see Fig. S2 in the supplementary material), suggesting insufficient amounts of both FGF9 and PGD2 in the pole segments without the center domain. Moreover, we performed rescue experiments by the exogenous addition of each signaling factor – FGF9 (50 ng/ml), IGF1 (50 ng/ml), PDGFB (50 ng/ml),

PGD2 (500 mM), HGF (100 ng/ml) and NGF (100 ng/ml) – to anterior and posterior segment explants at 12-14 ts. Of these signaling factors, the exogenous addition of FGF9 efficiently restored defective cord formation in both anterior and posterior segments (see Fig. S3 in the supplementary material). Next, to further clarify the role of FGF9 in the pole segments, we examined in detail the effects of exogenous FGF9 supply on *Sox9* expression and testis cord formation in segment cultures. After incubation for 12 to 18 hours, FGF9 addition induced high levels of *Sox9* expression in both anterior and posterior segments, showing a similar signal intensity to that in the middle segment of the same genital ridge (Fig. 5A). Such recovery of *Sox9* activation in the pole segments further induced maintenance of SOX9-positive cells and testis cord formation in the explants cultured for further 4 days after FGF9 treatment for 24 hours (Fig. 5B). The number of SOX9-positive cells tended to be increased in FGF9-treated anterior/posterior explants, although this effect did not prove statistically significant (lower panels in Fig. 5B). Moreover, exogenous supply of FGF9 significantly increased the capacity to induce well-defined testis cords in both anterior and posterior pole segments (Fig. 5C). These data suggest that FGF9 is one of the limiting factors in the capacity to induce testis cord formation in the anterior/posterior pole domains of developing XY gonads in vitro.

Fgf9 expression occurs in a center-to-pole manner immediately after the initial 6 hour time window, which leads to rapid activation of *Dusp6*, a downstream mediator of FGF signaling pathway, in the whole gonadal surface area

The spatiotemporal patterns of *Fgf9* expression were examined in developing XY gonads in vivo. Whole-mount in situ hybridization revealed that *Fgf9* expression was first detected in the central domain at 15 ts, and then its expression expanded toward the anterior and posterior poles at 18 ts (Fig. 6A). This clearly indicates that FGF9 secretion is restricted to the central domain in the early phase of testis differentiation in vivo, further suggesting a poleward diffusion of FGF9 toward the anterior and posterior pole ends from 15 ts.

Next, to monitor FGF9 signaling, the expression patterns of *Dusp6*, a downstream mediator of FGF signal (Eblaghie et al., 2003; Kawakami et al., 2003; Li et al., 2007), were examined in developing gonads in vivo. *Dusp6* expression showed sexual dimorphism: higher in the XY gonads than in the XX gonads at 18 ts (Fig. 6B). Most interestingly, its expression was rapidly upregulated in the whole gonadal surface area immediately after the onset of *Fgf9* activation in the central domain (n=8, '16 ts' in Fig. 6B). This is in contrast to incomplete poleward expansion of *Fgf9* expression (i.e. weak *Fgf9* signals in both anterior and posterior ends) even at 18 ts ('18 ts' in Fig. 6A). These findings suggest a very rapid poleward diffusion of FGF9 and subsequent synchronous activation of its downstream signaling pathway in the whole gonadal surface area immediately after the onset of the *Fgf9* expression.

In addition, *Dusp6* expression profiles were very similar to those of *Fgf9* expression in the segmented and intact whole explants of XY genital ridges (initiated at 12 ts, cultured for 16 hours): (1) lower expression levels in both anterior and posterior pole segments than in the middle segment of the same embryos; and (2) proper expression throughout the whole area of intact gonadal explants (n=4; Fig. 6C,D). Moreover, in the whole genital ridge explants initiated at 16-18 ts (16 hours culture), the treatment with FGF signal inhibitor, SU5402 (10 μM), repressed testis-specific high-level expression of *Dusp6* in the XY explants (n=5; Fig. 6E), whereas the exogenous addition of FGF9 (50 ng/ml) drastically upregulated *Dusp6* expression in XX explants (n=4; Fig. 6F). These data confirm that testis-specific *Dusp6* expression can be used to monitor the activation of the FGF signaling pathway in developing mouse gonads.

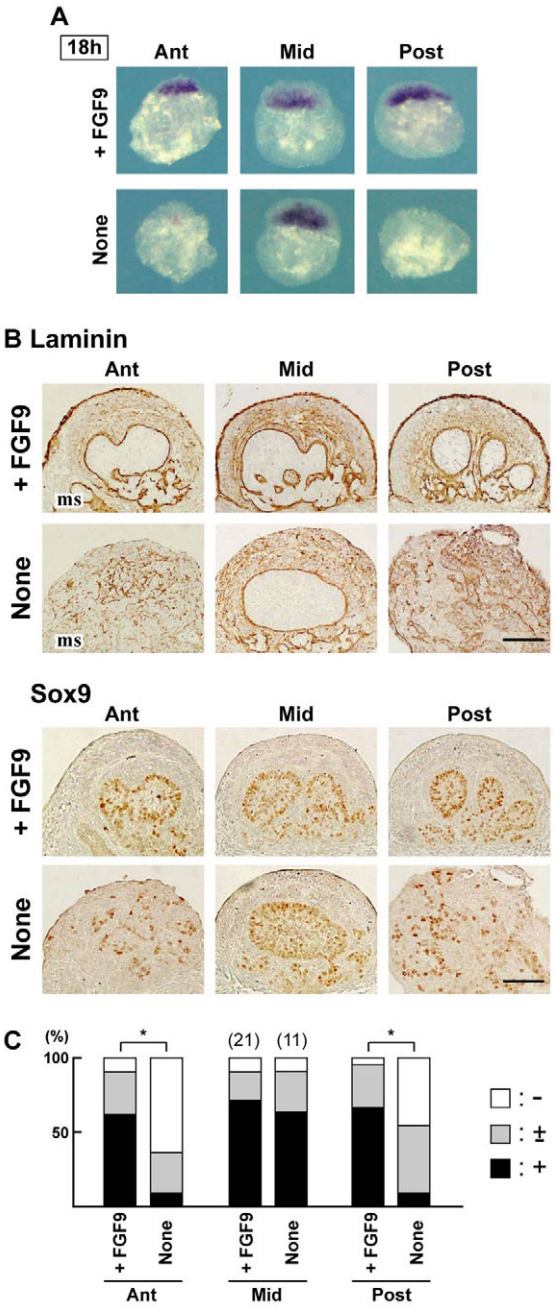

Fig. 5. Exogenous FGF9 supply rescues defective tubulogenesis of pole segment explants of XY genital ridges. (**A**) In situ hybridization analysis showing *Sox9* expression patterns in the segment explants (initiated at 12 ts) treated with or without FGF9 (50 ng/ml) after incubation for 18 hours. (**B**,**C**) Anti-laminin and anti-SOX9 immunostaining of the segment explants (12 ts) treated with or without FGF9 (50 ng/ml) for the initial 24 hours (total 5 days culture). In C, percentile graphs show relative numbers of the segment explants with no cord-like structure (white), slender cord-like structure (gray) or well-defined testicular cords (black) in the presence or absence of FGF9. Statistical analysis (Fisher's exact test) shows a significant recovery in testiculogenesis of the anterior/posterior segments by exogenous FGF9 addition (*P<0.05). ms, mesonephros. Scale bar: 100 μm.

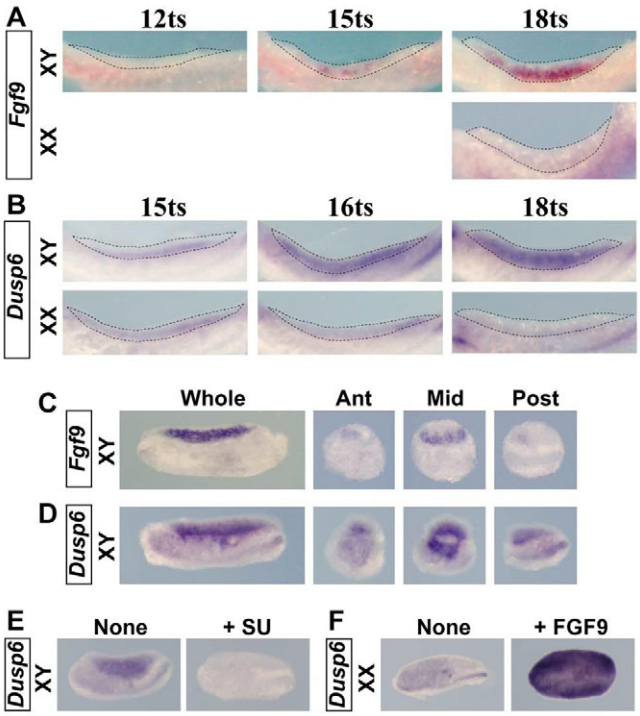

Fig. 6. Spatiotemporal expression patterns of *Fgf9* and *Dusp6*, a downstream mediator of FGF signaling pathway, in developing gonads in vivo and in vitro. (**A**,**B**) In situ hybridization analysis showing Fgf9 and Dusp6 expression profiles in developing gonads in vivo. Fgf9 expression occurs in a center-to-pole manner in developing XY gonads immediately after the 6 hour time window (note no appreciable Fgf9 signals in both anterior and posterior pole ends of the XY gonad at 18 ts). Dusp6 expression is rapidly upregulated in whole gonadal surface area of XY genital ridge at around 16 ts. The gonadal area is indicated by a broken line. (**C**,**D**) In situ hybridization analysis showing Fgf9 (C) and Dusp6 (D) expression in the whole control explants and the anterior, middle and posterior segment explants of XY genital ridges (initiated at 12 ts) after incubation for 16 hours. (**E**,**F**) In situ hybridization analysis showing Dusp6 expression profiles in the XY explants (initiated at 16 ts) treated with or without FGF signal inhibitor, SU5402 (10 μM) (E) and in the XX explants (16 ts) with or without FGF9 (50 ng/ml) (16 hours culture).

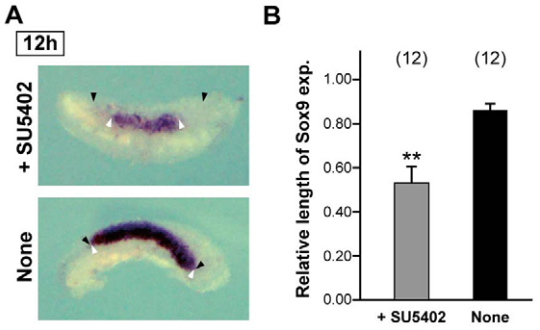

Fig. 7. Inhibition of FGF signaling represses a poleward expansion of *Sox9* expression in developing XY genital ridge in vitro. (**A**) In situ hybridization analysis showing *Sox9* expression in whole XY genital ridges explants (initiated at 12 ts) treated with or without FGF signal inhibitor SU5402 (10 μM) for 12 hours. Black arrowheads show anterior and posterior edges of the gonadal area, whereas white arrowheads indicate the edges of the *Sox9*-positive domain. (**B**) Quantitative analysis of the AP-axis length of *Sox9*-positive area per whole gonadal length in whole explants (initiated at 12-13 ts) treated with or without SU5402 (12 hours). In explants treated with SU5402, the *Sox9* expression domain is significantly shorter in length than those in non-treated XY explants (n=12; $^{**}P$<0.01).

Inhibition of FGF signaling represses poleward expansion of *Sox9* expression in developing XY gonads

To clarify the role of FGF9 signals in the poleward expansion of testiculogenic programs, we cultured intact whole XY genital ridges (12-13 ts) in the presence or absence of the FGF signal inhibitor SU5402 (10 μM) for 12 hours, and then examined the spatial patterns of the *Sox9*-positive domain in the explants. As expected, the center-to-pole expansion of *Sox9* expression in whole XY genital ridge explants was inhibited by the addition of SU5402, showing that the *Sox9*-positive domain is restricted to the central region of explants treated with SU5402 (Fig. 7A). Quantitative analysis of the relative expansion of the *Sox9*-positive area along the AP axis (i.e. the AP-axis length of *Sox9*-positive area per whole gonadal length) also confirmed that the length of the *Sox9* expression domain in XY explants treated with SU5402 was significantly shorter than that in non-treated control explants (n=12; Fig. 7B). These results indicate that the inhibition of FGF signaling blocks the poleward expansion of the *Sox9* expression domain into the anterior and posterior ends. This is consistent with the hypothesis that a poleward diffusion of FGF9 secreted from the advanced central domain contributes to proper testis cord formation in the anterior/posterior domains of developing XY gonads.

Reduced *Wnt4* activities do not affect the tubulogenic capacity of anterior/posterior pole domains in developing XY gonads

It is well established that WNT4 can act as an antagonistic signal against FGF9 signaling to promote the ovarian pathway in developing XX gonads (Kim et al., 2006). Finally, we endeavored to clarify a possible contribution of WNT4 signals to defective tubulogenic potency of the anterior and posterior segments in developing XY gonads.

The in vivo spatiotemporal patterns of *Wnt4* expression in developing XX and XY gonads (ICR background) were first examined by whole-mount in situ hybridization (Fig. 8A). In both XY and XX gonads, *Wnt4* expression was weakly detectable in the whole gonadal region at 12-13 ts, and a high level of *Wnt4* expression was detected in the anterior mesonephros throughout 12 to 18 ts. In developing XX gonads, *Wnt4* expression was gradually upregulated from around 15 ts, resulting in strong signals throughout the gonadal area at 18 ts (left panels in Fig. 8A). In XY gonads, its expression was clearly reduced in the central region at 15-16 ts, in contrast to weak signals detectable in both anterior and posterior poles at 15-18 ts (right panels in Fig. 8A). These results indicate that, in XY gonads, *Wnt4* expression is downregulated in a center-to-pole manner immediately after the 6 hour time window (12-14 ts), showing a pattern complementary to the poleward *Fgf9* upregulation from 15 ts (Fig. 6A).

We next examined *Wnt4* expression levels in segment explants of XY and XX genital ridges initiated at 12 ts (ICR background). In XX genital ridges, *Wnt4* expression was properly activated in all three segments after incubation for 18 hours (Fig. 8B). By contrast, in XY genital ridges, *Wnt4* expression was not detected in any of the three

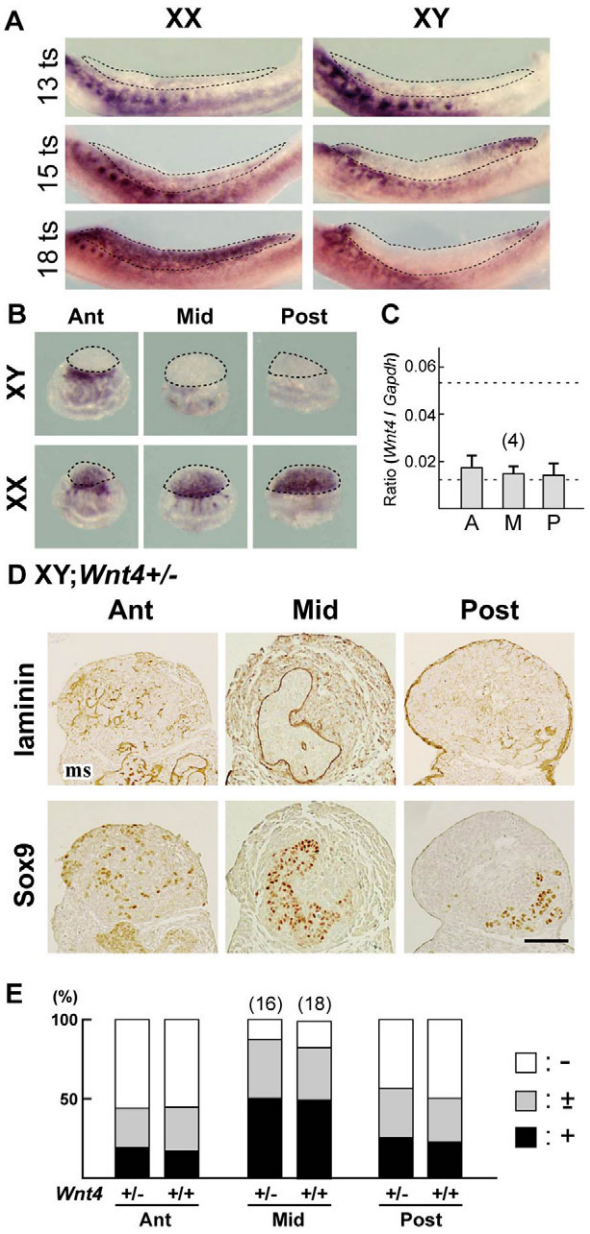

Fig. 8. Spatiotemporal patterns of *Wnt4* expression and tubulogenic activities of pole segment explants of XY *Wnt4*$^{+/-}$ genital ridges. (**A**) In situ hybridization analysis showing the onset of sexually dimorphic *Wnt4* expression immediately after the 6 hour time window. (**B**,**C**) In situ hybridization (B) and real-time RT-PCR (C) analyses showing *Wnt4* expression in the segment explants (initiated at 12 ts) after incubation for 18 hours. In C, vertical axis represents *Wnt4* expression level relative to *Gapdh* (the mean values ± standard error; n=4). Two horizontal broken lines indicate *Wnt4* expression levels in the XX ($5.34\pm0.32\times10^{-2}$) and XY ($1.21\pm0.20\times10^{-2}$) gonadal region at 21-22 ts, respectively. (**D**,**E**) Anti-laminin and anti-SOX9 immunostaining showing no appreciable influence of reduced *Wnt4* activities in defective tubulogenesis in the segment explants (initiated at 12-14 ts; 5 days culture). Scale bar: 100 μm.

segments, except in the anterior mesonephros. Quantitative real-time PCR analysis also showed no significant differences in *Wnt4* expression levels in the gonadal area among the three segments after incubation for 18 hours (Fig. 8C).

Finally, we isolated three segments of the genital ridge from XY *Wnt4*$^{+/-}$ or *Wnt4*$^{+/+}$ embryos at 12-14 ts (129SvJ/ICR mixed background), and then estimated their in vitro capacities to induce cord formation in 5 day cultures (Fig. 8D,E). The tubulogenic capacities of pole segments showed no significant differences between *Wnt4*$^{+/-}$ and *Wnt4*$^{+/+}$ genital ridges of XY embryos, indicating no appreciable influence of reduced *Wnt4* activity on tubulogenesis in both anterior and posterior segments (Fig. 8D,E). These data indicate that WNT4 signals are not directly involved in the center-to-pole expansion of tubulogenic capacity in developing XY gonads in this mouse strain background.

DISCUSSION

In this study, we provide the first direct evidence showing the supportive role of the advanced center domain in the proper progression of testis cord formation in the anterior and posterior poles. By performing segment and reconstruction culture assays of XY genital ridges at 12-14 ts, we demonstrated that the middle segment can support tubulogenesis in both anterior and posterior regions without any appreciable contribution of the gonadal parenchyma cells (Fig. 3C,D). Moreover, the present partition culture assay indicates a possible contribution of certain poleward diffusible factors that are secreted from the gonadal central domain to support testiculogenesis in the anterior and posterior pole domains at the early phase of testis differentiation (Fig. 4). During mouse testis differentiation, it is generally accepted that FGF9 promotes the maintenance of high-level *Sox9* expression in the supporting cell lineage via a positive-feedback loop (Colvin et al., 2001; Schmahl et al., 2004; Kim et al., 2006), which consequently leads to the proper organization of testis structures at later stages. The present study showed defective expression in both *Sox9* and *Fgf9* in the anterior and posterior pole explants initiated at 12-14 ts. Exogenous FGF9 addition could rescue defective formation of testis cords in these pole segments, whereas inhibition of FGF signaling prevented the poleward expansion of *Sox9*-positive domains into the anterior and posterior pole domains. As *Fgf9* expression is significantly higher in the central domain than in the poles at 15-16 ts, these data imply that the FGF9 signal acts as a poleward diffusible factor to support proper tubulogenesis in the pole domains in the developing XY gonad. The present expression analyses of *Fgf9* and *Dusp6* also suggest a rapid poleward diffusion of active FGF signaling in the whole gonadal area immediately after the onset of FGF9 secretion in the central domain (Fig. 6A,B). This theory is supported by recent studies that report classical ovotestis formation (with ovarian tissue in the poles) in XY gonads of *FGFr2*-null embryos (Kim et al., 2007; Bagheri-Fam et al., 2008).

A summary of our hypothesis of the role of poleward diffusion of FGF9 in testis formation in pole domains is represented schematically in Fig. 9. In brief, *Sry* is initially activated in the central domain at around 12 ts (11.0 dpc), which leads to *Sox9* expression at 13-14 ts and subsequent *Fgf9* expression at 15-16 ts (Fig. 9A). In the central domain, a positive-feedback loop between SOX9 and FGF9 is set up in a tissue-autonomous manner, resulting in the establishment of testis cord formation at later stages. In the pole regions, *Sry* starts to be activated at 13-14 ts, a delay of approximately 4 hours after the onset of *Sry* expression in the neighboring central domain. This *Sry* expression properly initiates low-level *Sox9* expression at 15-16 ts in pole regions. At the same time (15-16 ts), FGF9 is already expressed in the central domain, and presumably diffuses toward the anterior and posterior poles. Our data suggest that this poleward diffusion of FGF9 supports the establishment of a positive-feedback loop for SOX9 expression in

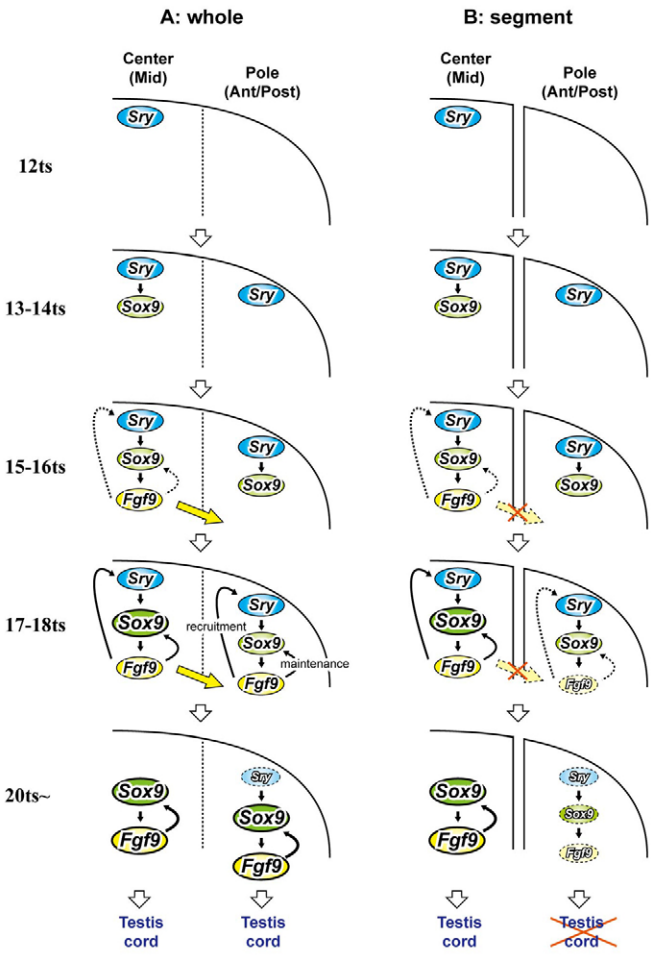

Fig. 9. A possible role of FGF9 signals as a diffusible conductor for directing center-to-pole expansion of the testiculogenic capacity in developing XY gonads. Schematic representation of intact whole (**A**) and segment (**B**) explants of XY genital ridges initiated at 12-14 ts, showing spatiotemporal patterns of testis initiation programs in the central (Mid) and anterior/posterior pole (Pole) domains along the AP axis. Both Sry expression and initial (low-level) Sox9 expression are properly activated in a center-to-pole manner in both whole and segment explants. A poleward diffusion of center-derived FGF9 (yellow arrows) is required for the establishment of high-level Sox9 expression in a positive-feedback manner in the pole domains.

the poles, leading to the maintenance of SOX9 expression, the recruitment of precursor cells and proper testis cord formation in the entire gonadal area. However, the removal or partition of the central domain before 15 ts disrupts the poleward diffusion of FGF9 toward the anterior and posterior poles (Fig. 9B), leading to insufficient FGF9 signaling in the poles. This probably causes defective maintenance of high *Sox9*-positive cells in the pole domains, resulting in the failure of proper testis cord formation at later stages.

Is it possible that the other soluble/diffusible factors (except for FGF9) are involved in the poleward expansion system of testiculogenesis in vivo as shown in Fig. 9? The answer is yes, if the soluble/diffusible factors can be upregulated immediately downstream of SRY/SOX9 and can support the establishment of high-level SOX9 expression in a positive-feedback manner. Among FGF family members except for FGF9, our preliminary experiments revealed that *Fgf10* expression is sex-dimorphically higher in the XY gonads than in the XX gonads at 18 ts (11.5 dpc) (see Fig. S4 and Table S1 in the supplementary material). Its expression also showed a higher level in the central domain than in the pole domains, indicating the functional redundancy of FGF9 and FGF10 in testiculogenesis. The present data also suggest that the PGD2 level is higher in the center domain than in the poles (see Fig. S2D in the supplementary material). It was shown that the PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells in testis differentiation (Monioto et al., 2009). Our recent study showed that some ECM components are involved in the positive-feedback regulation of SOX9 expression leading to proper tubulogenesis (Matoba et al., 2008). Therefore, it is possible for these candidate factors to contribute, albeit within the distinct ranges in their effective diffusion, to a rapid poleward expansion of testiculogenesis in developing XY gonads in vivo.

How significant is the center-to-pole expansion of testis initiation programs along the AP axis? It is likely that the center-to-pole expansion system with positive-feedback loops ensures a rapid female-to-male switching throughout the whole organ, a mechanism that may be especially important in the case of long and slender structures such as mouse gonadal primordium. It appears that FGF9 secretion from the gonadal center domain rapidly diffuses towards the poles to stabilize high-level SOX9 expression in pre-Sertoli cells (Colvin et al., 2001; Schmahl et al., 2004) and/or to recruit pre-Sertoli cell precursors (Karl and Capel, 1998; Schmahl et al., 2004), consequently leading to the rapid progress of testiculogenesis in the anterior and posterior pole domains. Such a mechanism would explain the lack of any appreciable lag in the timing of testis cord formation between the center and pole regions (i.e. synchronous tubulogenesis in the entire gonadal area) (Coveney et al., 2008), despite a delay of approximately 4 hours in initial *Sry* activation between these two positions (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001). Moreover, such a rapid progress of testiculogenic programs may be crucial for the inhibition of the onset of the female programs, including meiotic induction in germ cells located in the pole regions (Menke et al., 2003; Bullejos and Koopman, 2004; Bowles et al., 2006; Koubova et al., 2006). It is possible that sustained *Wnt4* expression in the poles and/or in the anterior mesonephros of the XY gonads (Fig. 8A) contributes to the onset of ovarian differentiation programs in the poles in developing XY gonads (Kim et al., 2006; Hiramatsu et al., 2009). However, we could not demonstrate any appreciable influence of reduced *Wnt4* activity in this study. Further studies to examine the timecourse expression profiles of *Fgf9* and *Wnt4* in developing XY ovotestes of B6-Ydom (Eicher et al., 1982; Bullejos and Koopman, 2005; Wilhelm et al., 2009), *Fgfr2*-null (Kim et al., 2007; Bagheri-Fam et al., 2008) and B6-MRL (Otsuka et al., 2008) embryos may provide some answers to these questions.

Acknowledgements
We thank Drs Patrick Tam and Jo Bowles for their comments on and critical reading of the manuscript, and Ms Itsuko Yagihashi for her secretarial assistance. The authors are grateful to Drs Hideyo Ohuch and Suzanne L. Mansour for providing in situ probes. This work was supported by Grants-in-Aid for Scientific Research (KAKENHI) on Priority Areas and Innovative Areas.

Competing interests statement
The authors declare no competing financial interests

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.040519/-/DC1

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Table S1. Primers used for the global expression profiles of all Fgf members (*Fgf1-23*)

Gene	Forward primer	Reverse primer
Fgf1	TCAACCTGCCTCTAGGAAAC	ACAGCTCCCGTTCTTCTTGA
Fgf3	TACCTGGCCATGAACAAGAG	GATCACTTGGGCTCTGCTTC
Fgf4	GGAGCACTTTCTCTCGATGG	GCACCCTTGGAAACTGATGT
Fgf5	TGTACTGCAGAGTGGGCATC	TCTCGGCCTGTCTTTTCAGT
Fgf6	TCAGTGGAACACACGAGGAG	CCCGTCCATATTTGCTCAGT
Fgf7	TTGACAAACGAGGCAAAGTG	TCCGCTGTGTGTCCATTTAG
Fgf9	CGGTACTATCCAGGGAACCA	GTATCTCCTTCCGGTGTCCA
Fgf10	CCTCGTCCTTCTCCTCTCCT	TTGCTGTTGATGGCTTTGAC
Fgf11	CAGAGGACACCAGCTCCTTC	GCTGCCTTGGTCTTCTTGAC
Fgf12	TTCCTGTAGGACTGCGTGTG	TTCCCCTTCATGATTTGACC
Fgf13	CCTCGGAACATTTCACACCT	AGAGACGCTTCTGCTCTTGG
Fgf14	TGCTGTACAGGCAACAGGAG	GTTGACTGGTTTGCCTCCAT
Fgf15	AGACGATTGCCATCAAGGAC	CTGGTCCTGGAGCTGTTCTC
Fgf16	CTGATCAGCATCAGGGGAGT	TCTCTCCGAGTCCGAGTGTT
Fgf17	GAGCAAAGACTGCGTGTTCA	TCCCTGACTACGTTTGGGAC
Fgf18	AAGCACATTCAAGTCCTGGG	AGCCCACATACCAACCAGAG
Fgf20	CGGCAGGATCACAGTCTCTT	CCCTGAAGATGCATTCAGAAG
Fgf21	ACCTGGAGATCAGGGAGGAT	GTCCTCCAGCAGCAGTTCTC
Fgf22	GAGATCCGTTCTGTCCGTGT	ACCAAGACTGGCAGGAAGTG

The RT-PCR data are shown in Fig. S4A. The primer sequences for *Fgf2*, *Fgf8* and *Fgf23* were reported previously (Hajihosseini and Heath, 2002).

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