Wilms tumor protein–dependent transcription of VEGF receptor 2 and hypoxia regulate expression of the testis-promoting gene Sox9 in murine embryonic gonads

Karin M. Kirschner, Lina K. Sciesielski, Katharina Krüeger, and Holger Scholz

From the 1Institut für Vegetative Physiologie and the 4Klinik für Neonatologie, Charité-Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

Edited by Xiao-Fan Wang

Wilms tumor protein 1 (WT1) has been implicated in the control of several genes in sexual development, but its function in gonad formation is still unclear. Here, we report that WT1 stimulates expression of Kdr, the gene encoding VEGF receptor 2, in murine embryonic gonads. We found that WT1 and KDR are co-expressed in Sertoli cells of the testes and somatic cells of embryonic ovaries. Vivo-morpholino–mediated WT1 knockdown decreased Kdr transcripts in cultured embryonic gonads at multiple developmental stages. Furthermore, WT1 bound to the Kdr promoter in the chromatin of embryonic testes and ovaries. Forced expression of the WT1(-KTS) isoform, which functions as a transcription factor, increased KDR mRNA levels, whereas the WT1(+KTS) isoform, which acts presumably on the post-transcriptional level, did not. ChIP indicated that WT1(-KTS), but not WT1(+KTS), binds to the KDR promoter. Treatment with the KDR tyrosine kinase inhibitor SU1498 or the KDR ligand VEGFA revealed that KDR signaling represses the testis-promoting gene Sox9 in embryonic XY gonads. WT1 knockdown abrogated the stimulatory effect of SU1498-mediated KDR inhibition on Sox9 expression. Exposure to 1% O2 to mimic the low-oxygen conditions in the embryo increased Vegfa expression but did not affect Sox9 mRNA levels in gonadal explants. However, incubation in 1% O2 in the presence of SU1498 significantly reduced Sox9 transcripts in cultured testes and increased Sox9 levels in ovaries. These findings demonstrate that both the local oxygen environment and WT1, which enhances KDR expression, contribute to sex-specific Sox9 expression in developing murine gonads.

Gonadal development is a unique process in which the undifferentiated genital ridge can give rise to two distinct organs, the testes and the ovaries. A single gene on the Y chromosome, Sry (sex-determining region of the Y chromosome) is both necessary and sufficient for testis formation (1). Sry is transiently expressed between 10.5 and 12.5 dpc2 in the genital ridge and pre-Sertoli cells of the male mouse embryo (2–4). In the absence of a Y chromosome, the bipotential gonads develop along the female pathway into ovaries. Activation of the R-spondin1/β-catenin pathway supports the differentiation of granulosa cells in the XX gonad (5). SRY and its major transcriptional downstream target gene Sox9 (sex-determining region Y-box 9) promote the differentiation of Sertoli cells, which in turn is a prerequisite for seminiferous cord formation in the testis (6, 7) (reviewed in Ref. 8). Thus, high Sox9 expression supports differentiation into male (XY) gonads.

The zinc finger transcription factor WT1 (Wilms tumor 1) is initially expressed in the somatic cells of the bipotential genital ridge. Later, WT1 expression becomes restricted to the Sertoli cells of the testis (9–11) and the granulosa cells of the ovary (12). Germ line WT1-deficient mice are embryonic lethal and lack gonads and kidneys due to apoptosis of their respective primordia (13). A role of WT1 in murine testis differentiation is confirmed by the disruption of seminiferous tubules and the loss of germ cells upon conditional WT1 deletion in Sertoli cells. Abnormal testis formation in these mice correlates with SOX9 depletion from WT1-deficient Sertoli cells after cessation of Sry expression (14). Development of the seminiferous tubules is closely linked to vasculogenesis in embryonic testes (15, 16). VEGF and its cognate receptor (KDR, also known as VEGFR2 or FLK-1) are essential for establishing the testis vasculature possibly by recruiting KDR-positive endothelial cells from the adjacent mesonephros, a transitory stage of the kidney (17, 18). At later developmental stages, KDR is co-expressed with WT1 in Sertoli cells and required for seminiferous cord formation in the testis (15, 16).

Mutations in the WT1 gene can give rise to dysgenesis of the gonads. Impaired WT1 function in XX gonads correlates with reduced follicle counts and decreased ovary size (19). WT1-related genital abnormalities in males range from hypospadias and cryptorchidism in WAGR individuals (Wilms tumor, aniridia, genitourinary abnormalities, mental retardation) to sex reversal and streak gonads in patients with severe Denys–Drash and Frasier syndromes (20–22). Frasier syndrome is caused by disruption of the donor splice site in intron 9 of the.

2 The abbreviations used are: dpc, days postconception; H3K4me3, histone H3 Lys-4 trimethylation; qPCR, quantitative PCR; nRbIgG, normal rabbit IgG.
WT1 gene, which normally leads to the insertion of the amino acids lysine, threonine, and serine (KTS) between the third and fourth zinc finger of the WT1 protein (23–25). Patients with the XY karyotype can develop female external genitalia and streak gonads due to a diminution of the +KTS/−KTS isomeric ratio (22). Whereas WT1(+KTS) proteins have a presumed role in RNA processing, WT1(−KTS) molecules act as transcription factors (26, 27) (reviewed in Ref. 28). Selective ablation of either WT1 isoform in mice revealed distinct functions of the + KTS/−KTS proteins during urogenital development (29).

Although WT1 has been implicated in the control of several critical genes in sexual development, including Sry, Sox9, and Amhr2 (14, 30, 31), its function during gonad formation is still incompletely understood. Considering the importance of normal vascularization for testis development (15, 16), one can speculate whether pro-vasculogenic WT1 target genes are involved in this process. Although WT1 has indeed been reported to enhance VEGF expression (32, 33), its role in the male gonads is still unknown. Because oxygen levels are usually lower in testes and ovaries. For this purpose, XX and XY gonads were isolated from murine embryos at 11.5, 12.5, and 13.5 dpc, respectively, and cultured ex vivo for 72 h in the presence of a sequence-matched antisense morpholino, which differed from a non-targeting siRNA. Efficient gene silencing was monitored by immunoblotting with an anti-WT1 antibody (Fig. 1B).

Assuming a direct stimulatory effect on transcription, one would expect that WT1 binds to regulatory regions in the Kdr gene. The latter possibility was tested by ChIP in embryonic XY and XX gonads, using the promoter of the Egr3 gene as a positive control (35). Enrichment of immunoprecipitated Kdr and Egr3 DNA was compared with a gene desert region with closed chromatin in the mouse genome (ChIP neg). A histone H3K4me3 antibody was used for the precipitation of open chromatin. Precipitation with WT1 antibody enriched the proximal promoter of the Kdr gene (Kdr F1/R1) ~8- and 14-fold in XY and XX gonads, respectively (Fig. 1D). This region contains two overlapping predicted binding sites matching the previously identified WT1 consensus sequence (35, 36) (Fig. 1C). A second genomic region including another putative WT1-binding element (Kdr F2/R2) in the 5′-UTR of the Kdr gene was not enriched with the WT1 antibody. These findings indicate that WT1 is necessary for normal Kdr expression and binds to the Kdr promoter in murine embryonic gonads.

**Results**

**WT1 increases Kdr expression and binds to the Kdr promoter in murine embryonic gonads**

Previous genome-wide expression analyses suggested that Kdr, the gene encoding vascular endothelial growth factor receptor 2, may represent a candidate downstream target of WT1.3 Considering the importance of WT1 for male and female gonad development (13, 14), we first explored whether WT1 is required for normal Kdr expression in embryonic testes and ovaries. For this purpose, XX and XY gonads were isolated from murine embryos at 11.5, 12.5, and 13.5 dpc, respectively, and cultured ex vivo for 72 h in the presence of a sequence-specific antisense vivo-morpholino to inhibit WT1 protein translation (Fig. 1A). All experiments were performed in a pairwise manner (i.e., the second gonad of each embryo was incubated with a mismatch vivo-morpholino, which differed from the WT1 morpholino in five nucleotides). Antisense inhibition of WT1 (WT1 vivo-morpholino) significantly decreased Kdr transcripts in XY gonads isolated at 11.5 and 12.5 dpc, but not at 13.5 dpc. In XX gonads, WT1 knockdown significantly reduced Kdr mRNA levels at all three developmental stages analyzed (Fig. 1A). Of note, in XX gonads, basal Kdr expression diminished after developmental time (Fig. 1A). WT1 and KDR are also expressed in embryonic kidneys. However, knockdown of WT1 did not significantly change Kdr transcript levels in murine embryonic kidney explants, suggesting a rather gonad-specific role for the regulation of Kdr expression by WT1 (supplemental Fig. S1). Embryonic gonads consist of multiple cell types and therefore do not allow one to distinguish between cell-autonomous and indirect effects of WT1 on Kdr expression. We addressed this issue by using the murine mesonephros-derived M15 cell line, which has robust WT1 protein levels. Transfection with WT1 siRNA reduced Kdr transcripts in M15 cells ~4-fold (p < 0.05) compared with treatment with non-targeting siRNA. Efficient gene silencing was monitored by immunoblotting with an anti-WT1 antibody (Fig. 1B).

**The WT1(−KTS) isoform stimulates KDR expression and binds to the KDR promoter**

Alternative pre-mRNA splicing gives rise to WT1 isoforms, WT1(+KTS) and WT1(−KTS), which differ in their chromatin-binding affinity. The WT1(−KTS) protein, which lacks the KTS (lysine-threonine-serine) amino acid insertion between zinc fingers 3 and 4, exhibits high DNA-binding affinity and functions as a transcription factor (reviewed in Ref. 28). By contrast, the WT1(+KTS) isoform has been reported to interact with RNA rather than DNA (26, 27). We used human osteosarcoma cells with inducible expression of either WT1(−KTS) or WT1(+KTS) protein to examine the effects of both WT1 isoforms on KDR expression (Fig. 2A). Induction of WT1(−KTS) by removal of tetracycline (−tet) from the culture medium increased KDR transcripts in UB27 cells more than 4- and 8-fold (p < 0.05) after 48 and 72 h, respectively. Overexpression of WT1(+KTS) in UD28 cells had no significant effect on KDR mRNA levels (Fig. 2A).

ChIP was performed to investigate whether WT1(−KTS) and WT1(+KTS) proteins bind to the promoter of the human KDR gene (Fig. 2C). Induced expression (−tet) of WT1(−KTS) in UB27 cells significantly enriched KDR promoter DNA using WT1 antibody for immunoprecipitation. Non-induced (+tet) UB27 cells, which contain no WT1(−KTS) protein, showed no enrichment (Fig. 2C). Intron DNA sequence of the ACTB gene lacking known WT1 binding sites served as a reference locus. An acetyl-histone H3 antibody was used as a control for immunoprecipitation of chromatin. Notably, incubation with WT1 antibody failed to enrich the KDR promoter in UD28 cells.

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3 K. M. Kirschner, L. K. Sciesielski, K. Krueger, and H. Scholz, unpublished observation.
WT1 stimulates Kdr expression in embryonic gonads

A. Kdr mRNA in cultured gonads of male (XY gonad) and female (XX gonad) mouse embryos with knockdown of WT1. The gonadal primordia were isolated from wild-type embryos at the indicated days dpc and incubated for 72 h in the presence of mismatch or Wt1 antisense vivo-morpholino. The gonads of each embryo were used in a pairwise manner (i.e., the explant incubated with the mismatch vivo-morpholino served as a control for the Wt1 vivo-morpholino–treated gonad). Kdr and Actin transcripts were measured by RT-qPCR. Data representing means ± S.E. are shown as -fold differences relative to 11.5 dpc XY gonads treated with mismatch vivo-morpholino. *, p < 0.05, paired t test. Representative immunoblots below the graphs demonstrate knockdown of WT1 in the morpholino-treated gonads.

B. Kdr mRNA levels in mesonephros-derived M15 cells transfected with either siRNA for WT1 knockdown (siWt1) or non-targeting siRNA (sicontrol). Kdr and Gapdh transcripts were quantified by real-time RT-qPCR. Values are shown as means ± S.E. (error bars), n = 4. Statistical significance is indicated by asterisks (paired t test; *, p < 0.05). WT1 and GAPDH proteins in M15 cells were detected by immunoblotting to determine knockdown efficiency, and representative data are shown.

C and D, ChIP was performed to detect WT1 protein bound to the 5'-flanking region of the Kdr gene. The drawing (C) delineates the promoter, 5'-UTR, and translational start of the mouse Kdr gene and allocates the qPCR primers (F for forward and R for reverse) used for DNA amplification of the Kdr core promoter (F1/R1) and the 5'-UTR (F2/R2). The sequence of predicted WT1 binding sites is indicated. D, specific antibodies were chosen for immunoprecipitation of WT1 and histone (H3K4me3) proteins associated with chromatin in XY and XX embryonic gonads at 13.5 dpc. Normal rabbit IgG (nRbIgG) was used as a negative control. Note that immunoprecipitation with WT1 antibody enriches Kdr promoter DNA (F1/R1) in XY and XX gonads, whereas the 5'-UTR (F2/R2) is not enriched. Primers binding to a transcriptional inactive region of the genome were used as a negative control (ChIP neg). Enrichment of the Egr3 promoter served as a positive control. Data are presented as means ± S.E. (error bars) of -fold increase relative to the ChIP neg/nRbIgG control. Statistical significance is indicated by asterisks (*, p < 0.05, paired t test compared with ChIP neg control; n = 7).

Figure 1. WT1 is necessary for normal Kdr gene expression in embryonic gonads and binds to the Kdr promoter. A, Kdr mRNA in cultured gonads of male (XY gonad) and female (XX gonad) mouse embryos with knockdown of WT1. The gonadal primordia were isolated from wild-type embryos at the indicated days dpc and incubated for 72 h in the presence of either mismatch or WT1 antisense vivo-morpholino. The gonads of each embryo were used in a pairwise manner (i.e., the explant incubated with the mismatch vivo-morpholino served as a control for the WT1 vivo-morpholino–treated gonad). Kdr and Actin transcripts were measured by RT-qPCR. Data representing means ± S.E. are shown as -fold differences relative to 11.5 dpc XY gonads treated with mismatch vivo-morpholino. *, p < 0.05, paired t test. Representative immunoblots below the graphs demonstrate knockdown of WT1 in the morpholino-treated gonads. B, Kdr mRNA levels in mesonephros-derived M15 cells transfected with either siRNA for WT1 knockdown (siWt1) or non-targeting siRNA (sicontrol). Kdr and Gapdh transcripts were quantified by real-time RT-qPCR. Values are shown as means ± S.E. (error bars), n = 4. Statistical significance is indicated by asterisks (paired t test; *, p < 0.05). WT1 and GAPDH proteins in M15 cells were detected by immunoblotting to determine knockdown efficiency, and representative data are shown. C and D, ChIP was performed to detect WT1 protein bound to the 5'-flanking region of the Kdr gene. The drawing (C) delineates the promoter, 5'-UTR, and translational start of the mouse Kdr gene and allocates the qPCR primers (F for forward and R for reverse) used for DNA amplification of the Kdr core promoter (F1/R1) and the 5'-UTR (F2/R2). The sequence of predicted WT1 binding sites is indicated. D, specific antibodies were chosen for immunoprecipitation of WT1 and histone (H3K4me3) proteins associated with chromatin in XY and XX embryonic gonads at 13.5 dpc. Normal rabbit IgG (nRbIgG) was used as a negative control. Note that immunoprecipitation with WT1 antibody enriches Kdr promoter DNA (F1/R1) in XY and XX gonads, whereas the 5'-UTR (F2/R2) is not enriched. Primers binding to a transcriptional inactive region of the genome were used as a negative control (ChIP neg). Enrichment of the Egr3 promoter served as a positive control. Data are presented as means ± S.E. (error bars) of -fold increase relative to the ChIP neg/nRbIgG control. Statistical significance is indicated by asterisks (*, p < 0.05, paired t test compared with ChIP neg control; n = 7).
WT1 stimulates Kdr expression in embryonic gonads

in the absence (−tet) and presence (+tet) of tetracycline (i.e., at high and low levels of WT1(+KTS), respectively) (Fig. 2C). These results demonstrate that only WT1(−KTS) and not WT1(+KTS) protein binds to the KDR promoter and stimulates expression of the KDR gene.

WT1 and KDR share an overlapping distribution in developing and adult gonads

WT1 expression in mature gonads is restricted to Sertoli cells of the testis and granulosa cells of the ovary (12). Assuming
that WT1 regulates Kdr expression in embryonic gonads, one would expect that both proteins are co-expressed during gonadal development. To test this, we performed double immunofluorescence staining with rabbit anti-KDR and mouse anti-WT1 antibodies in the gonads of rats. Rat tissue was chosen for technical reasons because of its suitability for the use with primary antibodies raised in mouse and rabbit. Embryonic XY and XX gonads were analyzed at the developmental stage 15.5 dpc, which corresponds to 14 dpc in mice (37). A clear overlapping expression of KDR and WT1 was seen in the seminiferous cords of embryonic XY gonads (Fig. 3). Whereas WT1 localized to the nuclei of Sertoli cells, KDR was detected in their cytoplasm and the basal membrane of the seminiferous cords (Fig. 3, a–d). A similar distribution of both proteins could also be observed in adult testes (Fig. 3, f–i). In the developing XX gonad, WT1 expression is restricted to the somatic cells and later the pregranulosa cells. KDR is present at the basement membrane of the pregranulosa cells enclosing the late stage primary oocytes in the inner zone of the developing ovary (Fig. 3, n). In adult ovaries, WT1 expression is observed in granulosa cells of the follicles (*). KDR is located at the outer boundary of the membrana granulosa (> in j). Incubation of the tissue sections with normal sera produced no specific staining signal (e, j, o, and t). Cell nuclei are stained with DAPI in some micrographs. Scale bars, 100 μm.

Figure 3. WT1 and KDR expression overlap in embryonic and adult gonads. Specific antibodies against WT1 (green) and KDR (red) were used to detect both proteins in the gonads of embryonic and adult rats. Immunolabeling identified Sertoli cells (>) and the coelomic epithelium (arrows in b and c) as the sites of WT1 expression in males. KDR is clearly co-expressed with WT1 in Sertoli cells (> in d and i). In the developing XX gonad, WT1 expression is observed in the somatic cells and later the pregranulosa cells. KDR is present at the basement membrane of the pregranulosa cells enclosing the late stage primary oocytes in the inner zone of the developing ovary (> in n). In adult ovaries, WT1 expression is observed in granulosa cells of the follicles (*). KDR is located at the outer boundary of the membrana granulosa (> in j). Incubation of the tissue sections with normal sera produced no specific staining signal (e, j, o, and t). Cell nuclei are stained with DAPI in some micrographs. Scale bars, 100 μm.

KDR inhibits the testis-promoting genes Sox9 and Sf1 in embryonic ovaries

Accumulating evidence suggests a role for VEGF signaling via KDR in gonadal development (15, 16). We therefore asked the question whether inhibition of KDR activity would impair the expression of testis- and ovary-promoting genes in XY and XX gonads. For this purpose, we analyzed the expression of Sox9, Sf1 (Nr5a1), Amh, Foxl2, Dax1, and Gata4 in 12.5-dpc XX and XY gonad cultures (Fig. 4A). Endogenous mRNA levels of Sox9, Sf1, and Amh were significantly higher in XY than in XX gonads, whereas XX gonads contained higher amounts of Fox2 and Dax1 transcripts than XY gonads. Gata4 transcript levels were not significantly different in embryonic testes and ovaries (Fig. 4A). To explore the importance of KDR for the expression of these genes in XY and XX gonads, we incubated the explants for 48 h with the KDR tyrosine kinase inhibitor SU1498, which has previously been found to inhibit testis cord formation in rats (15). SU1498 did not significantly change mRNA levels of Fox2 and Dax1 transcripts in XX gonads. Gata4 transcript levels were not significantly different in embryonic testes and ovaries (Fig. 4A). To explore the importance of KDR for the expression of these genes in XY and XX gonads, we incubated the explants for 48 h with the KDR tyrosine kinase inhibitor SU1498, which has previously been found to inhibit testis cord formation in rats (15). SU1498 did not significantly change mRNA levels of Fox2 and Dax1 transcripts in XX gonads. Gata4 transcript levels were not significantly different in embryonic testes and ovaries (Fig. 4A).

Kdr expression in embryonic gonads is stimulated by WT1 (Figs. 1–3). One can therefore predict that WT1 knockdown will reduce SU1498 activity due to down-regulation of its target, KDR. To test this, cultured gonads were treated with the KDR tyrosine kinase inhibitor SU1498 in the presence of the Wt1 vivo-morpholino. Consistently, Wt1 silencing abrogated the effect of SU1498 on Sox9, Sf1, and Gata4 mRNA levels in cultured testes and ovaries (Fig. 4B).
WT1 inhibits Sox9 in embryonic XX gonads through Kdr activation

Next, we explored how WT1 and KDR influence gonadal Sox9 expression. Maintenance of the tubular architecture in embryonic XY gonads requires activation of the testis-determining gene Sox9 by WT1 in Sertoli cells (14). Accordingly, Sox9 mRNA levels dropped in XY gonads (12.5 dpc) treated with the Wt1 vivo-morpholino (Fig. 5A). By contrast, WT1 knockdown increased Sox9 transcript levels in XX gonads ~2-fold (p < 0.005), and inhibition of KDR signaling with SU1498 prevented this effect (Fig. 5A). SU1498 also enhanced the inhibitory influence of WT1 silencing on Sox9 expression in developing XY gonads (Fig. 5, A and B). These findings indicate that WT1 regulates Sox9 in embryonic gonads in a sexually dimorphic manner; whereas it directly stimulates transcription of the testis-promoting gene Sox9 in Sertoli cells (14), WT1 represses Sox9 in XX embryonic gonads (Fig. 5). The latter effect is presumably mediated through a mechanism involving stimulation of Kdr expression by WT1 (Figs. 1–3).

KDR and WT1 are required for maintaining gonadal Sox9 mRNA levels in hypoxia

To further analyze the role of the KDR signaling pathway in Sox9 expression, we incubated gonadal explants with the KDR ligand VEGFA. The addition of recombinant VEGFA (500 ng/ml) to the gonad cultures for 48 h significantly reduced Sox9 transcripts in XX but not in XY gonads (Fig. 6A).

A physiological way to stimulate Vegfa expression is hypoxia, and tissue oxygen tensions are usually lower in embryonic than in adult tissues (reviewed in Ref. 34). Thus, to mimic the restricted oxygen supply in the fetus, we exposed gonadal explants to normobaric hypoxia. Incubation in 1% O2 for 24 h increased Vegfa transcripts in XY and XX gonads ~4- to 10-fold (p < 0.001), respectively (Fig. 6B). On the contrary, Wt1 mRNA levels were significantly reduced in both testes and ovaries at 1% O2 (Fig. 6C). Exposure to 1% O2 for 24 h did not significantly change Sox9 mRNA levels, neither in cultured embryonic testes nor in ovaries (Fig. 6D). The latter finding is physiologically relevant, given the fact that normal testis devel-
WT1 stimulates Kdr expression in embryonic gonads

A. Sox9 by WT1 in XX gonads depends on KDR signaling. A, gonadal explant cultures (XX and XY gonads, 12.5 dpc) were treated with WT1 vivo-morpholino, and the contralateral gonad of each embryo was incubated with mismatch vivo-morpholino as a control. Sox9 mRNA levels were measured by RT-PCR and normalized to Sdha transcripts. Data representing means ± S.E. (error bars) are shown as -fold differences compared with control (mismatch vivo-morpholino) XY gonads. Statistical significance is indicated by asterisks (paired t test; **, p < 0.01; *** p < 0.005; n = 13 (XY) and n = 12 (XX)).

B. Sox9 mRNA levels were measured by RT-qPCR. Data representing means ± S.E. (error bars) are shown as -fold differences compared with control (mismatch vivo-morpholino) XY gonads. Statistical significance is indicated by asterisks (paired t test; *, p < 0.05; n = 7 (XY) and n = 8 (XX)).

WT1 stimulates Kdr expression in embryonic gonads

B. Sox9 by WT1 in XX gonads depends on KDR signaling. A, gonadal explant cultures (XX and XY gonads, 12.5 dpc) were treated with WT1 vivo-morpholino, and the contralateral gonad of each embryo was incubated with mismatch vivo-morpholino as a control. Sox9 mRNA levels were measured by RT-PCR and normalized to Sdha transcripts. Data representing means ± S.E. (error bars) are shown as -fold differences compared with control (mismatch vivo-morpholino) XY gonads. Statistical significance is indicated by asterisks (paired t test; **, p < 0.01; *** p < 0.005; n = 13 (XY) and n = 12 (XX)).
WT1 stimulates Kdr expression in embryonic gonads

in embryonic testes (Fig. 5A) (14), we investigated whether down-regulation of WT1 in hypoxia (Fig. 6C) may account for the reduced Sox9 mRNA levels in XY gonads during KDR inhibition with SU1498. WT1 vivo-morpholino treatment indeed abrogated the decrease of Sox9 transcripts resulting from exposure of XY gonads to 1% O2 in the presence of SU1498 (Fig. 6, E and F).

Discussion

Differentiation of the bipotential genital ridge to either testis or ovary relies on the sex-specific activation of a few essential molecules. Among those, the transcription factor SOX9 directs the differentiation of progenitor cells toward the Sertoli cell lineage, which in turn is necessary for testicular cord formation (40, 41). It has been shown that WT1 stimulates Sox9 expression in embryonic XY gonads either directly or through indirect mechanisms (14, 29) (reviewed in Ref. 42). This is in agreement with the male-to-female sex reversal that occurs in mice with targeted deletion of either Sox9 or WT1 (29, 43, 44). In the present study, we used a vivo-morpholino to inhibit WT1 protein translation in ex vivo cultured murine embryonic gonads. We have recently shown that gonad morphology and sex-specific gene expression are well-preserved under these conditions (45). Our current findings confirm that WT1 contributes to the high Sox9 levels in developing testes beyond the stage of sex determination (Fig. 5A).

Unexpectedly, we observed that WT1 vivo-morpholino treatment significantly increased Sox9 transcripts in cultured ovaries (Fig. 5A). A similar up-regulation of Sox9 has recently been reported in the XX gonads of mice (13.5 dpc) with conditional WT1 deletion (46). These consistent findings indicate an inhibitory effect of WT1 on Sox9 expression in developing ovaries. Our following observations suggest that repression of Sox9 by WT1 in embryonic ovaries is indirect and involves KDR. The physiological KDR ligand VEGFA reduced Sox9 mRNA levels in embryonic XX gonads (Fig. 6A), whereas SU1498, a specific KDR tyrosine kinase inhibitor, increased Sox9 transcripts (Fig. 4A). Importantly, inhibition of KDR tyrosine kinase activity with SU1498 prevented the rise of Sox9 transcripts that was caused by WT1 knockdown in embryonic XX gonads (Fig. 5). Furthermore, WT1 silencing abrogated the stimulatory effect of SU1498 on gonadal Sox9 expression (Fig. 4, A and B). The failure of SU1498 to increase Sox9 mRNA levels in the absence of WT1 is probably due to down-regulation of its target, KDR (Figs. 1–3).

The close correlation between WT1 and KDR in developing testes and ovaries is certainly another interesting finding of this study. The following lines of evidence led us to conclude that WT1 stimulates transcription of the Kdr gene in embryonic gonads. First, antisense inhibition of WT1 significantly reduced Kdr transcripts in both embryonic testes and ovaries at various developmental stages (Fig. 1A). Second, WT1 protein bound to the Kdr promoter in the chromatin of embryonic gonads (Fig. 1, C and D). Third, WT1 silencing decreased Kdr mRNA levels also in mesonephros-derived M15 cells (Fig. 1B). Fourth, forced expression of the WT1(-KTS) isoform, which functions as a transcription factor (reviewed in Ref. 22), enhanced KDR expression in UB27 cells. In contrast, WT1(+KTS) protein, which presumably works through a post-transcriptional mechanism(s) (26, 27), did not significantly change KDR transcript levels in UD28 cells (Fig. 2A). Finally, chromatin immunoprecipitation demonstrated that WT1(-KTS) protein, but not the WT1(+KTS) molecule, binds to the KDR promoter (Fig. 2C). Actually, to our knowledge, this is the first study identifying a WT1 binding site in the chromatin of embryonic gonads, and the protocol described herein can be useful for genome-wide mapping of WT1 binding regions by ChIP-seq.

Transcriptional activation of the Kdr gene by WT1 is a novel finding that deserves more careful consideration. Previous studies identified VEGFA as a critical regulator of the sex-specific vascularization that is crucial for seminiferous cord formation in the developing testis (15, 16, 47). During this process, KDR-positive endothelial cells are recruited to migrate from the adjacent mesonephros into the testis, where they provide the vasculature and partition the gonad into domains for the seminiferous cords (16) (reviewed in Ref. 47). At later stages, KDR-expressing cells are dispersed around the seminiferous cords (15, 16). Notably, the KDR inhibitor SU1498, which we utilized in our study, reduced vascular development by ~90% and impaired seminiferous cord formation in organ cultures of rat testis (15). Our present findings indicate that besides regulating vasculogenesis in embryonic gonads, the VEGF/KDR pathway is also important for sex-specific gonadal gene expression (Fig. 4A).

Preserving appropriate gene activities despite the variable influence of endogenous and external signals is a prerequisite for normal gonad development. In this regard, one must recall that physiological oxygen concentrations are markedly lower in embryos than in postnatal individuals (reviewed in Ref. 34). Hypoxia enhanced Vegfa expression but reduced WT1 transcript levels in both XY and XX gonads (Fig. 6, B and C). This opposite regulation of Vegfa and WT1 in hypoxia may allow for a balanced, sex-specific Sox9 expression in the gonads independent of changing oxygen conditions. Consistently, hypoxia did not affect Sox9 expression in otherwise untreated gonads (Fig. 6D). However, hypoxia reduced Sox9 transcripts in cultured testes and increased Sox9 mRNA levels in embryonic ovaries during inhibition of KDR signaling (Fig. 6, E and F).

Taken together, our findings indicate that hypoxia, WT1, and VEGF/KDR jointly control gonadal Sox9 expression in a sex-specific manner. WT1 enhances Sox9 expression in the developing testis, whereas activation of the Kdr gene by WT1 provides an inhibitory signal for Sox9 in the embryonic ovary. We suggest that this intricate interplay between WT1 and KDR signaling protects Sox9 expression against the confounding influence of low oxygen conditions in the developing gonads.

Experimental procedures

Animals

All procedures were performed according to the Animal Protection Law guidelines and approved by the legal authorities represented by the Landesamt für Gesundheit und Soziales Berlin (LAGeSo). Experiments were conducted under permit T0308/12 issued by the LAGeSo. Mouse breeding pairs (C57/BL6/J strain) were mated in the in-house animal facility in com-
pliance with the local laws. The morning of vaginal plugs was considered as 0.5 dpc. Sex determination of the embryos was performed by PCR amplification of the Y chromosomal gene Kdm5d from genomic DNA using the following primers: CTGAAAGCTTTTGGCTTTTGGAG (mKdm5d-F) and CCACACCCAGCTTTGGAGCCAC (mKdm5d-R) (48).

Cell culture

The UB27 and UD28 cell lines, which express the murine WT1 (−KTS) and WT1 (+KTS) protein isoforms, respectively, under control of a tetracycline-repressed promoter, were the gift of Dr. Christoph Englert (49). Both cell lines as well as the murine mesonephros-derived M15 cells (26) were cultured as described elsewhere (26, 49).

ChIP assay

Mouse embryonic gonads (two embryonic gonads per immunoprecipitation sample) were isolated and fixed with 1% methanol-free paraformaldehyde in PBS for 10 min on ice. Fixation was stopped by the addition of glycine to a final concentration of 250 mM. Gonads were washed three times with PBS, and homogenized with Ultraturrax IK10. The tissue pellet was resuspended in hypotonic lysis buffer (20 mM HEPES-NaOH (pH 7.5), 250 mM sucrose, 3 mM MgCl₂, 0.2% Nonidet P-40, 3 mM β-mercaptoethanol) and incubated for 10 min on ice. The tissue pellet was dissolved in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)) before sonication in a Bioruptor Plus (Diagenode). Antibody-coupled magnetic beads (Magna HiSens Protein A/G, Millipore) were incubated with tissue lysate at 4 °C overnight. The following antibodies were used: anti-WT1 (sc-192, lot B0413, Santa Cruz Biotechnology, Inc.), anti-H3K4me3 (C15410030, Diagenode), normal rabbit IgG (sc-2027, Santa Cruz Biotechnology). Beads were washed once with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl), lithium chloride buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)), and with TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Incubation in elution buffer (1% SDS, 0.1 M NaHCO₃) and 15 min at room temperature removed the protein-DNA complexes from the beads. The DNA cross-link was released by the addition of NaCl at a final concentration of 300 mM and 3-h incubation at 65 °C. Proteins were digested by the addition of 75 µg/ml Proteinase K (Qiagen) for 1 h at 45 °C. DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1) precipitation. Primers used for qPCR are listed in Table 1.

UB27 and UD28 cells were kept for 72 h in the presence or absence of tetracycline (1 µg/ml) to either inhibit or stimulate WT1 (−KTS) and WT1 (+KTS) protein expression, respectively. ChIP assays were performed with ~4 × 10⁶ cells as described elsewhere (50). Normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) instead of anti-WT1 antibody (sc-192, lot L0109, Santa Cruz Biotechnology) was used as a negative control. Immunoprecipitation with anti-acetyl-histone H3 (06-599, Upstate Biotechnology, Inc.) antibody was performed for internal quality validation. Primers used for qPCR are listed in Table 1.

Organ culture experiments

 Gonads and kidneys were excised from embryos of timed pregnant mice. The matched organs of each embryo were cultured and treated for 48 or 72 h, respectively. ChIP assays were performed with WT1 (−KTS) and WT1 (+KTS) protein expression, respectively. ChIP assays were performed with anti-WT1 (sc-192, lot B0413, Santa Cruz Biotechnology) antibody was performed for a pairwise comparison between cultured explants from a single donor (36, 45). Gonads were cultured and treated for 48 or 72 h, as indicated. For inhibition of KDR signaling, explants were kept in the presence of 10 µM SU1498 (Abcam), which was dissolved as stock solution in DMSO. The final DMSO concentration in the culture medium was 0.01%. Control experiments

### Table 1

| Primer   | Sequence                  |
|----------|---------------------------|
| mKdr-F   | TGCCGATTTCCTCCATCCCTCCC  |
| mKdr-R   | GCTTGAATATCCTGACCTCATACG  |
| mGAPDH-F | GCACACTCCCTTACCTGGCACTCA  |
| mGAPDH-R | TTTTCCTCCATTCTGCAATAGTG   |
| mAcdb-F  | CCTGACACAGACTCTTCTCTTC    |
| mAcdb-R  | GCTGACCTCCCTTACCTGAGGATATG |
| mSdha-F  | ACCGCGTACCTCATCAATCTGG    |
| mSdha-R  | GTCACACAGACTCTTCTTCTCTTC  |
| mSor9-F  | CTGCGGCCGACACTGAGGAGT    |
| mSor9-R  | GCTGACCTCCCTTACCTGAGGATATG |
| mSFl-F   | GAGGTGGGCTTCCCTCTGCTCTCTT |
| mSFl-R   | GAGTTTGCGGCCCCAAAATGTAATT |
| mAnlh-F  | GGCCGTGGCTAGGGGACGTTG    |
| mAnlh-R  | CCCGGTGGAAACCTCAGGCTTCT   |
| mFod2-F  | AAGCGGCGTCTTGTCCATTGAG    |
| mFod2-R  | AGTTTGCGGCCCCAAAATGTAATT |
| mMcmx1-F | TGCGTTAGGTGCCCCAAAGAT    |
| mMcmx1-R | AGCGTCTCCTGCTCTTCCCA      |
| mMApple4-F | GAGCCGCGGAGGACACTCTACCTGG |
| mMApple4-R | ACCGCGTACCTCATCAATCTGG    |
| mVega-F  | CTGCGGCCGACACTGAGGAGT    |
| mVega-R  | GCTGACCTCCCTTACCTGAGGATATG |
| mVTr-F   | TAGGAGATGGGGCTCCAAAGTCC   |
| mVTr-R   | GAGATGGGGCTCCAAAGTCC     |
| mWt1-R   | TAGGACAGTGTGTTCTTCCACTTC |
| hKDR-F   | CTGACCGGACACTGAGGAGT     |
| hKDR-R   | GCTGACCGGACACTGAGGAGT    |
| hGAPDH-F | ACAGCTACGACACTGATCTCTT   |
| hGAPDH-R | GGAGGCGTCTTCCCTCTTCG     |

### WT1 stimulates Kdr expression in embryonic gonads

WT1 stimulates Kdr expression in embryonic gonads

![Primer Table](image-url)
**WT1 stimulates Kdr expression in embryonic gonads**

were performed with DMSO vehicle. Recombinant murine VEGFA (Peprotech) was used at 500 ng/ml for stimulation of KDR signaling.

**Preparation of RNA and RT-qPCR**

Total RNA was isolated from primary tissues and cells with the RNeasy Micro Kit (Qiagen) and from permanent cell lines with the TRizol LS reagent (Invitrogen). First-strand cDNA synthesis was carried out using oligo(dT) primers and Superscript™ III reverse transcriptase (Invitrogen). Quantitative PCR amplification was performed with the FastStart Universal SYBR Green Master (Rox) (Roche Applied Science) and the StepOnePlus™ system (Invitrogen) as described in detail elsewhere (51). The PCR primers for quantitative RT-PCR are listed in Table 1. Relative transcript levels were obtained by subtracting the threshold cycle (Ct) value of the housekeeping gene (β-Actin or Gapdh) from the corresponding Ct value of the gene of interest. Differences in mRNA levels were calculated according to the term 2ΔΔCt.

**Immunohistochemistry**

Immunofluorescent stainings were performed on rat tissues as described in detail elsewhere (50, 52, 53). Briefly, rat embryos (15.5 dpc) and adult organs were flash-frozen in tissue-Tek (O.C.T. compound (Sakura Finetek) and sectioned on a cryostat. Frozen sections (8 μm) were thawed and subsequently blocked for 5 min at room temperature in serum-free DakoCytomation protein block (catalogue no. X0909, Dako). Double immunostainings for WT1 and KDR were performed using the following primary antibodies diluted in ready-to-use antibody diluent (Zymed Laboratories Inc.): rabbit polyclonal anti-KDR (diluted 1:100; sc-315, Santa Cruz Biotechnology) and mouse monoclonal anti-WT1 (diluted 1:300; F6, MAB4234, Millipore). For visualization of the bound primary antibodies, Cy3-AffiniPure donkey anti-rabbit IgG (diluted 1:300; catalogue no. 711-165-152, Jackson ImmunoResearch, Dianova) and Alexa Fluor® 488-AffiniPure donkey anti-mouse IgG (diluted 1:90; catalogue no. 715-545-151, Jackson ImmunoResearch, Dianova) were used. The cell nuclei were counterstained with DAPI. Micrographs of double stainings were taken with a digital camera connected to a confocal microscope (Leica DM 2500, Leica Microsystems) utilizing LAS AF Lite software (Leica Microsystems).

**SDS-PAGE and immunoblotting**

Cells and tissues were lysed in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 4% urea, 1% (w/v) SDS, 7.5 mM DTT) and processed as described elsewhere (52). Incubation with anti-WT1 antibody (C19, catalogue no. sc-192, diluted 1:400; Santa Cruz Biotechnology) in 2.5% nonfat milk (Roth) in TBS/Tween was performed overnight at 4°C. After washing with TBS/Tween, the antibodies were detected with a peroxidase-coupled IgG (donkey anti-rabbit IgG-HRP, catalogue no. sc-2313, diluted 1:20,000; Santa Cruz Biotechnology), and the reaction products were visualized with WesternBright Sirius HRP substrate (Advanta) following the user’s manual. Equal protein loading was assessed with an ACTIN antibody (anti-ACTIN clone C4, catalogue no. MAB1501R, diluted 1:6,000; Millipore) after stripping of the membranes with 0.2 M NaOH for 10 min.

**Statistics**

Two-tailed paired t test and analysis of variance with Tukey’s post-hoc test were performed as indicated to reveal statistical significance. p values < 0.05 were considered significant unless otherwise stated.

**Author contributions**—K. M. K. and H. S. designed the work and wrote the manuscript. Most of the experiments were done by K. M. K. with the help of all contributing co-authors. Data analysis and interpretation were performed by K. M. K. assisted by H. S. Important intellectual contributions at an advanced stage of the manuscript came from L. K. S. and K. K.. The submitted version of the manuscript was approved by all authors.

**Acknowledgments**—We gratefully acknowledge the expert technical assistance of Ulrike Neumann and Ursula Kastner.

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