Tex 19 Paralogs Exhibit a Gonad and Placenta-Specific Expression in the Mouse

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Abstract. We have previously suggested that TEX19, a mammalian-specific protein of which two paralogs exist in rodents, could be implicated in stem cell self-renewal and pluripotency. We have established here the expression profiles of *Tex19.1* and *Tex19.2* during mouse development and adulthood. We show that both genes are coexpressed in the ectoderm and then in primordial germ cells (PGCs). They are also coexpressed in the testis from embryonic day 13.5 to adulthood, whereas only *Tex19.1* transcripts are detected in the developing and adult ovary as well as in the placenta and its precursor tissue, the ectoplacental cone. The presence of both *Tex19.1* and *Tex19.2* in PGCs, gonocytes and spermatocytes opens the possibility that these two genes could play redundant functions in male germ cells. Furthermore, the placental expression of *Tex19.1* can explain why *Tex19.1* knockout mice show embryonic lethality, in addition to testis defects.

Key words: Embryo, Gonad development, *In situ* hybridization, Placenta, Spermatogenesis

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T*ex19* (Testis expressed gene 19) was initially identified as testis specific through a cDNA subtraction strategy [1]. *Tex19* is restricted to mammals, is present as a unique gene in humans, and has been duplicated in the mouse and rat giving rise to the paralogs *Tex19.1* and *Tex19.2*. Through protein comparison in 11 mammalian species, two highly *Tex19*-conserved domains named MCP and VPTEL domains have been characterized [2]. However, none of them shares homologies with known proteins, therefore preventing any functional prediction. Interestingly, an N-Ter cysteine was recently found to be bound to Ubr2, which may help *Tex19* stabilization [3].

Analysis of *Tex19.1* knockout (KO) mice highlights a critical role for *Tex19* in spermatogenesis, where it seems to act as a repressor of transposable elements. It was also reported that about half of the homozygous pups are missing, suggesting a function of *Tex19.1* in survival during the perinatal period [4, 5]. It was further shown that *Tex19.1* is expressed by different pluripotent stem cells such as embryonic stem (ES), embryonic germ (EG) and embryonic carcinoma cells (ECC) and that the pattern of *Tex19.1* expression almost parallels that of *Oct4*, a canonical marker of cell pluripotency. Altogether, these latter observations suggest that *Tex19.1*,

as well as its human ortholog, could be a new factor involved in the maintenance of self-renewal or pluripotency of stem cells. Up to now, the expression patterns of *Tex19.1* and *Tex19.2* have been essentially analyzed by RT-PCR [2], thus limiting the amount of information on the cell types expressing these genes.

With the aim of helping to evaluate *Tex19.1* and *Tex19.2* functions, we decided to precisely determine their expression patterns at the cellular level by *in situ* hybridization (ISH) of whole mounts and/or histological sections from E (embryonic day) 7.5 to adulthood. Our results indicate notably that *Tex19.1* and *Tex19.2* expression patterns mostly overlap in male mice and thus that functional redundancy between these two genes could attenuate the *Tex19.1* KO phenotype.

Materials and Methods

Embryos and gonads

CD1 mice were housed in an animal facility licensed by the French Ministry of Agriculture (agreement No. B67–218–5) and animal experiments were supervised by MM (agreements No. 67–62) who is qualified in mouse experimentations, in compliance with the European legislation on care and use of laboratory animals. The morning of finding a vaginal plug was designated day 0.5 of pregnancy. Adults were at least 8 weeks old.

In situ hybridization (ISH) on histological sections and whole mount *in situ* hybridization (WISH) analyses

ISH and WISH were performed with *Oct4* (312 bp), *Tex19.1* (487

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Table 1. Primer sequences and RT-PCR conditions for mouse *Tex19.1*, *Tex19.2*, *Oct4* and β -actin genes

Gene	Primer name	Primers sequence	Annealing temperature (Ann T)	Fragment size	PCR program
Mouse <i>β-actin</i>	AHX 298 (sense)	CAGAGCAAGAGAGGTATCCT	58 C	507 bp	96 C: 5 min 94 C: 30 sec 58 C: 30 sec 72 C: 10 sec 72 C: 10 min } × 35 cycles
	AHX 299 (antisense)	CATCTCCTGCTCGAAGTCTA			
Mouse <i>Tex 19.1</i>	AHW 110 (sense)	TTGTGGTACTGACTACCACC	58 C	487 bp	95 C: 15 min 94 C: 30 sec Ann. T C: 1 min 30 sec } × 35 cycles 72 C: 1 min 30 sec 72 C: 10 min
	AHW 111 (antisense)	AAGGAGCACGAGTAACGAAC			
Mouse <i>Tex 19.2</i>	AHW 112 (sense)	AGGGACTGGTGCTTGTACCA	58 C	414 bp	
	AHW 113 (antisense)	ACAGGGCACGAACTGAGCAA			
Mouse <i>Oct4</i>	AER 61 (sense)	CACGAGTGGAAAGCAACTCA	58 C	519 bp	
	EBR 136 (antisense)	CTCGAACCACATCCTTCTCT			
Mouse <i>Tex 9.1</i> and <i>Tex19.2</i> (Duplex PCR)	AHB 124 (sense)	TTGGAGCAAGTGTGAAGCTG	60 C	566 bp (Tex 19.1)	
	AHB 125 (antisense)	ATCCAGGGGTACAGCTTCCT		496 bp (Tex 19.2)	

bp) and *Tex19.2* (414 bp) probes. The *Oct4* probe, which strongly labels PGCs and germ cells (GCs) within gonads, is helpful to follow PGC migration and gonad location of GCs during embryogenesis and adulthood. WISH was performed on whole embryos at developmental stages when gonads could not be dissected and on gonads when the ISH signal of sections was too weak to be informative. RNA sense and antisense digoxigenin-labeled probes were synthesized using a Dig RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions.

For WISH analyses, embryos or dissected gonads (with mesonephros attached) were collected and fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.2, at 4 C, for 4 h (for embryos until E7.5 and gonads) or over night (for E8.5 and older embryos). After rinsing the samples twice in PBS containing 0.1% Tween (PBT), the samples were dehydrated in methanol diluted in PBT (from 25% to 100%) and stored at -20 C. After rehydration in PBT, the samples were treated as previously described [6] with a modified washing solution (2X SSC, 50% formamide and 1% SDS) and 0.05g/ml polyvinyl alcohol (Sigma P1763, Saint-Louis, MO, USA).

Sections destined for ISH were prepared as described [2]. In brief, ten-micrometer-thick sections of fresh-frozen tissues were postfixed for 10 min in PFA in PBS at 4 C before the prehybridization step. Hybridization was performed over night at 70 C with the appropriate denatured probes in a humid chamber. Then sections were washed, and the probe was revealed. Sections were counterstained either with 0.01% safranin or with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

RNA analyses

Embryos and gonads dissected for RNA extraction were either dry frozen or kept in the appropriate amount of RLT buffer (Qiagen, Hilden, Germany) according to the manufacturer in liquid nitrogen

before keeping them at -80 C. Total RNA was prepared after thawing on ice using a Midi or Mini extraction kit (Qiagen), according to material quantity and the manufacturer's instructions. Reverse transcription of total RNA was performed using Superscript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Simplex PCR amplifications of *Oct4*, *Tex19.1* and *Tex19.2* genes from gonad and embryo cDNAs were performed using a PCR program with a master mix Kit (Qiagen). Duplex PCR amplification of *Tex19.1* and *Tex19.2* from extraembryonic tissue cDNAs was performed using a master mix kit (Qiagen). All β -actin and *Oct4* PCR amplifications were performed with Roche Taq polymerase (Roche Diagnosis) and a master mix kit (Qiagen), respectively. All experiments were performed according to manufacturers' instructions. Conditions are described in Table 1. *Oct4* was used as a positive control for the presence of germ cells, and β -actin was used as a positive control for RNA extraction.

Results

The expression pattern of *Tex19.1* and *Tex19.2* was analyzed by RT-PCR and/or ISH in whole mounts or histological sections of embryos and gonads using probes specific for each gene transcript. *Oct4* was used both as a marker of primordial germ cells (PGC) [7] and of ES cell pluripotency [8].

Expression of *Tex19.1* in the placenta and sexually undifferentiated embryos

Tex19.1 was strongly expressed in the ectoplacental cone at E7.5 as assessed by ISH (C, Fig. 1A and 3A) as well as in the placenta at E9.5 and E10.5 and, to a lesser extent, at E13.5 and E17.5, as assessed by RT-PCR analysis (Fig. 1C). At E13.5, expression of *Tex19.1* in the placenta was restricted to spongiotrophoblast cells in the junctional zone (J, Fig. 1B) and to some cells in the trabeculae

Fig. 1. Expression of *Tex19.1* in the ectoplacental cone (E7.5) and placenta (E9.5 to E17.5). (A and B) ISH (purple signal) in histological sections at E7.5 and E13.5. C, ectoplacental cone; CP, chorionic plate; D, maternal decidua; E, ectoderm; EN, endoderm; J, junctional zone of the placenta; L, labyrinthine zone of the placenta; M, mesoderm. Bar in B: 80 μ m (A) and 300 μ m (B). (C) RT-PCR analyses of *Tex19.1* and β -actin gene expression in the placenta at E9.5, E10.5, E13.5 and E17.5.

in the labyrinthine zone (L, Fig. 1B).

Embryonic expression of *Tex19.1*, is present in the E3.5 blastocyst [2] and was found at E6.5 in the present study (Fig. 2). At E7.5, *Tex19.1* transcripts were present in the ectoderm (E, Fig. 1A and 3A). At E8.5, *Tex19.1* was detected in the embryo, exclusively in a region of the posterior hindgut known to contain *Oct4*-positive PGCs (arrows in Fig. 3D, E [9]), and accordingly, a faint signal was observed by RT-PCR at this age (Fig. 2). *Tex19.1* was detected in the hindgut at E9.5 (arrow in Fig. 3F) and then in the nascent gonad at E11.5 (G, Fig. 3I). From E8.5 to E11.5, *Tex19.1* expression almost paralleled that of *Oct4*, with the exception of the ectoplacental cone, where *Oct4* transcripts were absent (Fig. 3). This observation strongly suggests that, similarly to *Oct4*, *Tex19.1* is expressed in migrating primordial germ cells and then in gonocytes prior to the onset of sexual differentiation.

Expression of *Tex19.1* in the developing and adult testis

Using ISH, *Tex19.1* expression was detected in seminiferous cords of the embryonic and fetal testis at E12.5, E13.5 and E16.5 (SC, Fig. 4A, D and G). In histological sections, ISH signals revealed expression in gonocytes (G) at E16.5 and E18.5 (Fig. 5A, B, G and H), with the staining looking like that of *Oct4*, a germ cell-specific marker during development and after birth (Fig. 5E, F, K and L). At this stage, gonocytes are located in the center of seminiferous cords and have a clear nucleus. After birth, *Tex19.1* expression was still restricted to gonocytes (G) at P1 (Fig. 5M and N) and at P5 (Fig. 5S

Fig. 2. RT-PCR analyses of *Tex19.1*, *Tex19.2*, *Oct4* and β -actin gene expression in embryos. Blank: PCR run with water. Adult stomach (St) and testis (T) were used as negative and positive controls, respectively.

Fig. 3. ISH analysis of *Tex19.1*, *Tex19.2* and *Oct4* expression in sexually undifferentiated embryos. ISH signals are purple. C, ectoplacental cone; E, ectoderm; H, head folds; G, gonad; TA, tail. Arrows point to the hindgut. Embryos in A–C, D and E, F–H and I–K were photographed at the same magnifications, respectively.

and T). *Tex19.1* staining follows cell margination, which is specific of germ cells at this stage of development. The same process is observed with *Oct4* ISH, as shown in Fig. 5 (G, Fig. 5Q, R, W and X). In the adult, *Tex19.1* transcripts were present in pachytene (P) spermatocytes (Fig. 6A–D). In contrast, *Oct4* expression in sexually mature testes was specifically detected in round spermatids (R) at stages VII and VIII of the seminiferous epithelium cycle (Fig. 6I and J), as previously described [10].

Expression of *Tex19.1* in the developing and adult ovary

Tex19.1 expression was detected in the embryonic and fetal ovary at E12.5, E13.5 and E16.5 (O, Fig. 7A, D and G), and in the prepubertal ovary at P1 (OO, Fig. 7J). At all these developmental stages, the *Tex19.1* expression profile was similar to that of *Oct4* and was thus restricted to oocytes (OO, Fig. 7J). Likewise, in the

Fig. 4. ISH analysis of *Tex19.1*, *Tex19.2* and *Oct4* expression in the developing testis from E12.5 to E16.5. M, mesonephros; SC, seminiferous cords; T, testis. ISH signals are purple in whole-mount ISH. Pictures A–C, D–F and G–I were photographed at the same magnifications, respectively.

adult ovary, expression of *Tex19.1* and *Oct4* corresponded to that of oocytes (OO, Fig. 7 M–P).

Expression of *Tex19.2*

Tex19.2 was undetectable in the placenta and in its precursor, the ectoplacental cone, at all studied developmental stages (C, Fig. 3B and data not shown). *Tex19.2* transcripts were present in the ectoderm of E7.5 embryos and undetectable in E8.5 embryos by RT-PCR (Fig. 2) and ISH (data not shown). At E9.5, *Tex19.2* was expressed in the hindgut of the embryos in a pattern similar to that of *Tex19.1* and *Oct4* (arrow, Fig. 3G, compared with F and H). No expression was observed at E11.5 (e.g., G in Fig. 3J) or in the testis at E12.5 (T, Fig. 4B). In the male gonad from E13.5 to E16.5, *Tex19.2* expression was restricted to the seminiferous cords (SC, Fig. 4E and H). In histological sections, ISH revealed its expression in gonocytes at E16.5 (G, Fig. 5C and D), which were located in the center of seminiferous cords at this stage, with the same staining as *Oct4*, a germ cell-specific marker (G, Fig. 5E and F). At E18.5, *Tex19.2* expression appeared almost restricted to Sertoli cells (S, Fig. 5I and J), as previously described [2], with weak expression remaining in gonocytes (G, Fig. 5I and J). At E16.5 and E18.5, Sertoli cells were located at the periphery of seminiferous cords and had a darker nucleus compared with gonocytes, which were centrally located and had a clearer nucleus. The gonocyte-specific staining was confirmed by the staining observed in *Oct4* ISH sections (G, Fig. 5E, F, K and L). Soon after birth, at P1 and P5, *Tex19.2* transcripts were detected mainly, if not exclusively, in Sertoli cells (S, Fig. 5O, P, U and V), although some weak expression in gonocytes was not possible to rule out (G, Fig. 5O, P, U and V). At these stages, Sertoli cell cytoplasm occupies the majority of the central part of the tubules, and germ cells are marginating, with a clearer nucleus. Indeed, gonocyte-specific staining but not Sertoli cell staining is

Fig. 5. ISH analysis of *Tex19.1*, *Tex19.2* and *Oct4* expression in the developing testis from E16.5 to P5. G, gonocyte; S, Sertoli cells. Histological sections were counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI), and the ISH signals were converted into a red false color in the merged pictures (B, H, N, T, D, J, P, V, F, L, R and X, respectively). Note that Sertoli cell cytoplasm, which contains *Tex 19.2* transcripts, occupies the center of the seminiferous cords at postnatal days 1 and 5. Bar in X: 80 μ m (A–X).

observed in *Oct4* ISH sections (Fig. 5Q, R, W and X). In adult testes, *Tex19.2* transcripts were restricted to pachytene spermatocytes, with some variation in the intensity of the ISH signal depending on the stage of the seminiferous epithelium cycle (P, Fig. 6E–H).

In the female gonad after E12.5, almost no expression was observed except at E13.5 and E16.5, at which time a very faint signal could be observed (Fig. 7B, E and H) that was similar to that of *Oct4* at these stages (Fig. 7F, I). After birth, no signal could be seen in ISH sections (Fig. 7K) or in the adult ovary, as found by RT-PCR [2].

Discussion

The *Tex19* gene was first identified as a unique gene and with a male germ cell-specific expression [11]. Subsequently, we showed

Fig. 6. ISH analysis of *Tex19.1*, *Tex19.2* and *Oct4* expression in the adult testis. E, elongated spermatids; P, pachytene spermatocytes; R, round spermatids; ST, seminiferous tubules. ISH signals are purple. The histological sections displayed in A, C, E, G and I were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and the ISH signals were converted into a red false color in the merged pictures (B, D, F, H and J, respectively). Bar in J: 80 μ m (A, B, E, F, I and J) and 30 μ m (C, D, G and H).

that *Tex19* is duplicated in the mouse and rat, giving rise to *Tex19.1* and *Tex19.2* paralogs [2]. A preliminary study of their expression patterns [2] shows that *Tex19.1* transcripts are present in the placenta but are otherwise germ cell specific in both the male and female. *Tex19.1* knockout mice display a reduced male fertility and a perinatal lethality affecting about half of the mutants [4]. The placenta, whose potential defects could account for the lethal phenotype of the *Tex19.1* mutants, was however not analyzed. Furthermore, the two *Tex19.1* knockout studies [3, 4] do not address a possible functional

Fig. 7. ISH analysis of *Tex19.1*, *Tex19.2* and *Oct4* expression in the developing (A–L) and adult (M–P) ovary. F, ovarian follicle; M, mesonephros; O, ovary; OO, oocyte. ISH signals are purple. The histological sections displayed in J–L were counterstained with safranin. The histological sections displayed in M and O were counterstained with DAPI, and ISH signals were converted into a red false color on the merged pictures (N and P, respectively). Note that A–C, D–F and G–I were photographed at the same magnifications, respectively. Bar in P: 40 μ m (J–L) and 30 μ m (M–P).

redundancy between *Tex19.1* and *Tex19.2*. This redundancy can only operate providing that the two genes display overlapping expression domains or if the knockout of a given paralog induces an ectopic expression of the remaining one. Therefore, there was a need to fully address the expression pattern of *Tex19.1* and to establish that of *Tex19.2*.

In the present study, expression of these genes was analyzed by RT-PCR and/or ISH from E6.5 to adulthood. *Tex19.1* is present in blastocysts [2] and at E6.5. *Tex19.2* is not expressed before E7.5. At E7.5, *Tex19.1* and *Tex19.2* show overlapping expression in the ectoderm, whereas only *Tex19.1* is expressed in the ectoplacental cone. At E9.5, *Tex19.1* and *Tex19.2* expression is limited to cells which, according to their specific localization in the hindgut and their expression of *Oct4*, are known to represent migrating PGCs. When testes differentiate, *Tex19.1* and *Tex19.2* expression also overlap in the seminiferous cords of the fetal testis until E16.5. From E18.5 to soon after birth, a differential expression is observed between germ and somatic cells, as previously described [2]. *Tex19.1* expression is then specific to germ cells, whereas *Tex19.2* is mainly expressed in somatic cells. In adults, both genes are expressed in pachytene spermatocytes. The expression patterns of *Tex19.1* and *Tex19.2* reported in the present study are in accordance with the finding that (1) severely affected adult testes of *Tex19.1* knockout mice lack all

postmetiotic cells, their most advanced cell type being pachytene spermatocytes [4], and (2) spermatocytes lacking *Tex19.1* display synapsis defects [4, 5]. Moreover, the variability in the phenotype of *Tex19.1* knockout males, favors the existence of a redundancy between *Tex19.1* and *Tex19.2* at some stages of development. The reason why both genes are temporarily expressed in different cell types remains an open question. In particular, the existence of common or divergent regulation factors for *Tex19.1* and *Tex19.2* expression remain to be investigated.

Tex19.1, but not *Tex19.2*, is also expressed in the early developing female gonad and oocytes as well as in the ectoplacental cone and placenta. The birth of a healthy individual not only involves an appropriate germ cell development but also the integrity of the placenta. Many genes have been ascribed a specific expression either in somatic or germ cells in the gonads and in placental cell populations. This is the case for *Esx1* [11] and a cluster of genes representing the *Rhox* homeobox gene family, which includes *Rhox5* (or *Pem*) [12], *Rhox6* (or *Psx1*) [13] and *Rhox9* (also known as *Psx2* or *Gpbox*) [14]. Three of these genes have been successfully ablated in the mouse. The *Esx1* knockout results in embryonic growth retardation due to placental defects but in fertile adults [15]. The phenotype of *Rhox* knockout mice show either a subfertility [16] or no pathological phenotype [17]. The mild or absent placental pathologies in *Rhox* null mutants could be explained by a redundancy between these genes, as they share a high degree of homology and present overlapping expression patterns. The proximity of the two genes, *Rhox5* and *Rhox9*, has precluded the generation of mice carrying a *Rhox5/Rhox9* double knockout and explains why this redundancy could not be challenged.

Similar to *Esx1* or members of the *Rhox* homeobox gene family, *Tex19.1* presents expression limited to male and female gonads and the placenta. Our data indicate that in the male mouse, *Tex19.1* and *Tex19.2* expression domains mainly overlap during development and adulthood. In their knockout study of *Tex19.1*, Ollinger *et al.* [4] detected a slight upregulation of *Tex19.2* expression in the mutant testes that could eventually explain the variability of the *Tex19.1* knockout phenotype. Our present study supports this scenario, as both genes are expressed in PGCs, gonocytes and pachytene spermatocytes. Kuntz *et al.* [2] have shown that *Tex19.2* is specifically expressed in somatic cells of the testis at E18.5, which we confirm here and also observe from birth to P5. Surprisingly, we detected this expression in germ cells in adult testes. The stage-dependent expression of *Tex19.2* may reflect a specific need for *Tex19* in somatic cells during testis development. Besides, the ectoplacental cone and placental *Tex19.1* expression that we show here could also explain why pups were missing in knockout animals [4]. Therefore, only a double *Tex19.1/Tex19.2* inactivation in the same mouse to impair functional redundancy between these paralogs could allow for determination of their exact functions.

Although the present study focuses solely on transcripts, it is noteworthy that we showed previously that *Tex19.1* and *Tex19.2* proteins were localized in the nucleus of NIH3T3 cells. In contrast, Ollinger *et al.* [4] and Yang *et al.* [3] reported a cytoplasmic localization of the *Tex19.1* protein in ES cells [4] and in male germ cells undergoing meiosis [3, 4]. Differences in the cell types used in these experiments and/or in the epitopes recognized by antibodies

generated in different laboratories may explain these discrepancies.

Acknowledgments

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