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# TGFβ signaling in male germ cells regulates gonocyte quiescence and fertility in mice

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ABSTRACT

During testis development, proliferation and death of gonocytes are highly regulated to establish a standard population of adult stem spermatogonia that maintain normal spermatogenesis. As Transforming Growth Factor beta (TGFbeta) can regulate proliferation and apoptosis, we investigated its expression and functions during testis development. We show that TGFbeta2 is only expressed in quiescent gonocytes and decreases gonocyte proliferation *in vitro*. To study the functions of TGFbeta2, we developed conditional mice that invalidate the TGFbeta receptor type II in germ cells. Most of the *knock-out* animals die during fetal life, but the surviving adults show a reduced pool of spermatogonial stem/progenitor cells and become sterile with time. Using an organ culture system mimicking *in vivo* development, we show higher proportions of proliferating and apoptotic gonocytes from 13.5 dpc until 1 dpp, suggesting a reduction of germinal quiescence in these animals. Conversely, a 24-hour TGFbeta2-treatment of explanted wild-type testes, isolated every day from 13.5 dpc until 1 dpp, increased the duration of quiescence.
These data show that the TGFbeta signaling pathway plays a physiological role during testis development by acting directly as a negative regulator of the fetal and neonatal germ cell proliferation, and indicate that the TGFbeta signaling pathway might regulate the duration of germ cell quiescence and is necessary to maintain adult spermatogenesis.



## Introduction

During testicular development, the germ lineage passes through cycles of proliferation and apoptosis. In the mouse, after sexual differentiation occurring at 11.5 days post-conception (dpc), fetal male germ cells (named gonocytes) actively proliferate until 16.5 dpc. Between 16.5 and 19.5 dpc, gonocytes are quiescent, and after birth they resume mitosis. In parallel, numerous gonocytes during both the fetal and neonatal phases of proliferation will die by apoptosis, whereas none of them will die during the quiescent phase (Olaso and Habert, 2000). These stages are essential for the establishment of a normal spermatogenesis. After birth, the gonocyte population gives rise to type A spermatogonia, which will differentiate during spermatogenesis to produce sperm (de Rooij and Grootegoed, 1998).

This fetal and neonatal period of testis development has been largely studied in rodents, especially in the rat (Beaumont and Mandl, 1963) (Hilscher et al., 1974) (Boulogne et al., 1999) and mouse (Vergouwen et al., 1991) (Nagano et al., 2000). Nevertheless, the mechanisms regulating the establishment of the quiescence of germ cells and resumption of their cell cycle at birth are not yet clear. Many pathways have been explored and some results suggest that genes such as *Pten*, *P27*, *P15* or *P53* could be involved in the germ lineage regulation (Beumer et al., 1999) (Moreno et al., 2001) (Moreno et al., 2002) (Kimura et al., 2003) (Western et al., 2008). Because the establishment of normal spermatogenesis is a fundamental process, there probably exists a redundancy of several pathways to preserve germ cell integrity in case of a defect in the main regulation system.

One of the pathways known to be involved in cell regulation is the Transforming Growth Factor beta (TGFbeta) signaling. *TGFbeta* (Moses et al., 1981) (Roberts et al., 1981) belongs to a superfamily of genes including also the bone morphogenetic proteins (BMPs), the anti-Müllerian hormone (AMH), the growth and differentiation factor (GDF), the distantly related glial cell line-derived neurotropic factor (GDNF), the activins / inhibins (Kingsley, 1994), and Nodal (Schier, 2003). TGFbeta signaling controls numerous cellular processes including cell proliferation, differentiation and apoptosis, both during embryogenesis and adulthood. Three isoforms of TGFbeta have been currently described in mammals: TGFbeta1, beta2 and beta3.

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They initiate signaling via the transmembrane type I (TβRI) and type II (TβRII) receptors serine/threonine kinase on the cell surface (Derynck, 1994) (Kingsley, 1994). The signal is then transmitted to the Smad proteins (Attisano and Wrana, 2002). A third type of TGFbeta receptor (TβRIII), also called betaglycan, has been described as an accessory receptor, and is known to facilitate the binding of TGFbeta (especially TGFbeta2) to TβRII (Lopez-Casillas et al., 1993). *TβRII* was identified for the first time in 1992 (Lin et al., 1992) and appears to be the most important signaling receptor (Wrana, 1998). TGFbeta ligands must bind to TβRII before TβRI is recruited. Once the holo-complex is formed, the kinase domain from TβRII trans-phosphorylates the GS domain of TβRI, which in turn phosphorylates Smad proteins (Lin et al., 2006).

Expression of TGFbeta isoforms and receptors has been described in the rat during testis development and it was shown that targets of TGFbeta signaling seem to be Leydig cells and gonocytes (Teerds and Dorrington, 1993) (Gautier et al., 1994) (Olaso and Habert, 2000). Additionally to immunohistochemical studies, functional assays performed *in vitro* demonstrated a potential effect of TGFbeta on steroidogenesis, cord formation and gonocyte behavior (Gautier et al., 1997) (Olaso et al., 1998) (Cupp et al., 1999). Other *in vitro* data showed an anti-proliferative effect of TGFbeta1 on mouse primordial germ cells (PGCs) (Godin and Wylie, 1991) (Richards et al., 1999) and an apoptotic role of TGFbeta on rat fetal and neonatal gonocytes during the proliferative phases of testis development (Olaso et al., 1998). Nevertheless, a physiological role of TGFbeta signaling in gonocyte development has never been demonstrated *in vivo*. The best way to establish such a physiological role for TGFbeta signaling would be the use of transgenic models.

Models of mice lacking TGFbeta isoforms or receptors have been generated (Dickson et al., 1995) (Oshima et al., 1996) (Sanford et al., 1997) (Nguyen and Pollard, 2000) (Larsson et al., 2001) (Stenvers et al., 2003) (Memon et al., 2008). To avoid a probable redundancy between isoforms, the best way is to disrupt one of the receptors. It is known that each type II receptor is specific for its subfamily, unlike type I receptors. This means that deletion of *TβRII* would disturb the TGFbeta signaling only, and not the signaling of the other family members. Null mutation of *TβRII* in all cells from early embryonic stages leads to growth retardation and severe anemia between 10.5 and 12.5 dpc (Oshima et al., 1996). In order to overcome this limitation, we undertook to generate *TβRII* conditional knock-out mice (*condKO*), via the Cre/Lox system, to target the disruption specifically in germ cells.

With this model, we demonstrated that TGFbeta signaling in germ cells is physiologically involved in the regulation of male gametogenesis as a direct anti-proliferative and anti-apoptotic factor. This study also showed that TGFbeta might regulate gonocyte quiescence phase and is fundamental in the initiation and maintenance of adult spermatogenesis.

## Materials and methods

### *Mice*

All animal studies were conducted in accordance with *the guidelines for the care and use of laboratory animals* of the French Ministry of Agriculture. Animals were housed under controlled photoperiod conditions (lights on 08.00-20.00) and were supplied with commercial feed and tap water *ad libitum*. Males were caged with females overnight and the day after mating was counted as 0.5 day post-conception (dpc). Birth occurs at 19.5 dpc, which was counted as 0 day post-partum (dpp).

The generation of the $T\beta RII^{flox/flox}$ mice has been previously described (Chytil et al., 2002).

To knock out *TβRII* in the PGCs, these floxed mice (kindly provided by H. Moses, Vanderbilt University, USA), maintained on a C57BL/6 background, were mated with *TNAP-Cre* mice (kindly provided by A. Nagy, Samuel Lunenfeld Research Institute, Canada), maintained on a hybrid 129 Sv/C57BL/6 background. Male $T\beta RII^{+/flox}$/*TNAP-Cre* mice were then crossed with female $T\beta RII^{flox/flox}$ mice, as ectopic recombination may be avoided when the *TNAP-Cre* locus is transmitted from the male (Lomeli et al., 2000) (Suzuki et al., 2001).

### *Genotyping*

Animals were genotyped by PCR analysis of genomic DNA from tail biopsy. To facilitate genotyping of mice, primer pairs were designed for detection of both wild-type (WT) and floxed (FL) alleles (8w-a/LA-LoxP: 5'-TAAACAAGGTCCGGAGCCCA-3' / 5'-ACTTCTG-CAAGAGGTCCCCT-3'), or for deleted (DEL) allele (8w-a/mSAr: 5'-TAAACAAGGTCCGGAGCCCA-3' / 5'-AGAGTGAAGCCGTGGTAGGT-GAGCTTG-3') (Chytil et al., 2002). The Cre-specific primer pair was as follows: CreForw / CreRev: 5'-GATGCAACGAGTGATGAGGTTCGC-3' / 5'-ACCCTGATCCTGGCAATTTCGGC-3'.

### *Immunohistochemistry*

Testes were fixed overnight at 4 °C with Bouin's fluid (2% (w/v) picric acid, 7% (v/v) formaldehyde, 3.8% (v/v) acetic acid), dehydrated and embedded in paraffin.

Sections were rehydrated and boiled twice for 5 min in a 10 mM sodium-citrate solution for antigen retrieval (Shi et al., 1991). Endogenous peroxidase activity was blocked with 0.3% (v/v) $H_2O_2$ in methanol, and non-specific binding sites were blocked by incubating the sections with 5% (w/v) bovine serum albumin in PBS. Subsequently, the slides were incubated overnight at 4 °C with the following primary antibodies: anti-TGFβ1 (1:50; sc-146, Santa-Cruz Biotechnology Inc., Santa Cruz, CA); anti-TGFβ2 (1:50; # 905-582, Assay Designs); anti-TGFβ3 (1:200; sc-82, SantaCruz Biotechnology Inc.); anti-TβRI (1:50; sc-398, SantaCruz Biotechnology Inc.); anti-TβRII (1:50; sc-220, SantaCruz Biotechnology Inc.), anti-TNAP (1:50; sc-23430, Santa Cruz Biotechnology Inc.), and anti-PLZF (1:50; sc-22839, Santa Cruz Biotechnology Inc.). After washing in PBS, slides were incubated at room temperature with a secondary biotinylated antibody (ABC peroxidase staining kit; Vector Laboratories Inc., Burlingame, CA) diluted 1:200. The avidin-biotin complex reaction was performed according to the manufacturer's protocol. To visualize bound antibodies, sections were covered with 3, 3'-diaminobenzidine (DAB; substrate kit for peroxidase, Vector Laboratories Inc.). Negative control sections were treated as described above, except that primary antibody was omitted during the procedure and replaced by 5% bovine serum albumin in PBS.

### *Organotypic cultures*

Mouse fetal testes were cultured on Millicell CM (Fischer Scientific Labosi, Elancourt, France) filters (pore size 0.4 mm) as previously described (Livera et al., 2006). Testes from embryos were collected at 13.5 dpc and the mesonephros was removed. Briefly, for each animal, each testis was placed on a filter floating on medium in tissue-culture dishes and incubated at 37 °C in a humidified atmosphere containing 95% air and 5% $CO_2$. The culture medium used was a mix of Dulbecco's modified Eagle's medium (DMEM) and Nutrient Mixture F-12 Ham's (HAM'sF12) (1:1) (Gibco, Cergy Pontoise, France). No serum, hormone or growth factor was added to the medium. Testes were cultured for one day (corresponding to 14.5 dpc *in vivo*), four days (corresponding to 17.5 dpc *in vivo*), six days (corresponding to 0 dpp *in vivo*), or seven days (corresponding to 1 dpp *in vivo*).

To measure testicular responsiveness to TGFβ, tissue-culture dishes had been previously siliconed with Sigmacote (Sigma-Aldrich Lyon, France) to avoid TGFβ binding to the plastic dishes. One testis was cultured for 24 hours in a control medium and the contra-lateral testis