Developmental Expression and Gene Regulation of Insulin-like 3 Receptor RXFP2 in Mouse Male Reproductive Organs

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ABSTRACT

The mutations of testicular insulin-like 3 (INSL3) hormone or its receptor RXFP2 cause cryptorchidism in male mice. Here we have examined Rxfp2 gene expression at different stages of embryonic and postnatal mouse development in male reproductive tissues employing quantitative RT-PCR and several RXFP2-specific antibodies directed toward different parts of the RXFP2 protein. Receptor expression was markedly increased after birth and was readily detectable in the epididymis, Leydig cells, and germ cells of the testis. The strongest expression was detected in adult mouse cremaster muscle. INSL3 treatment increased cell proliferation of embryonic gubernacular and TM3 embryonic Leydig cells, implicating active INSL3-mediated autocrine signaling in these cells and identifying TM3 as a novel in vitro model to study the effects of RXFP2 signaling. We generated Tg(Rxfp2cre-cre)Ala (Rxfp2-iCre) transgenic mice expressing improved Cre recombinase (iCre) under the control of the 2.4-kb mouse Rxfp2 promoter. The iCre was expressed in the gubernacular ligament at E14.5, indicating that this promoter is able to drive Rxfp2 gene expression during transabdominal testis descent. We demonstrated that the transcription factor Sox9, a known male sex determination factor, is expressed in mouse embryonic gubernacula and upregulated human, but not mouse, promoter luciferase reporter constructs. In conclusion, we have determined the developmental expression profile of INSL3 receptor employing newly characterized RXFP2 antisera and a novel Rxfp2-iCre transgenic mouse model. We determined the promoter region capable of providing the gubernacular-specific expression of Rxfp2. Analysis of RXFP2 promoter identified Sox9 as a new transcriptional enhancer of human gene expression.

INTRODUCTION

Testicular descent is an integral part of the male differentiation process. It begins in mice at embryonic day 14.5 (E14.5), along with the outgrowth of the caudal genital ligament, the gubernaculum, and postnatal testis descent into the scrotum [1]. The onset of testicular descent coincides with the differentiation of testicular Leydig cells and the Leydig-specific expression of the insulin-like 3 factor, INSL3. The targeted mutation of the Insl3 gene causes high intra-abdominal cryptorchidism and male infertility in mice [2, 3]. It was established that first transabdominal descent is androgen independent and can be induced even in females exclusively by overexpression of Insl3 [4, 5]. Transcriptional regulation of the Insl3 gene was studied in cell co-transfection experiments in vitro [6, 7], in several mouse mutants [6, 7], and in laboratory rodents upon treatment with various endocrine disruptors [8–11].

Less is known about the regulation and the expression pattern of the INSL3 receptor, RXFP2, a relaxin family peptide receptor 2, also called LGR8, leucine-rich repeat-containing GPCR, G protein-coupled receptor, or GREAT, G protein-coupled receptor affecting testicular descent [12–15]. Similarly to Insl3, the disruption of the Rxfp2 gene leads to a cryptorchid phenotype in Rxfp2-mutant mice [12, 13]. At E14.5, the Rxfp2 gene is expressed most prominently in gubernacular ligaments [16]. In the testis, Rxfp2 expression, as evaluated by in situ hybridization, was preferentially localized to the germ cells within the seminiferous tubules [17]. Rxfp2 mRNA was also found in several other organs and tissues, suggesting an involvement of INSL3 signaling in functions other than reproduction. For example, Rxfp2 expression was detected in the kidney, suggesting that INSL3/RXFP2 signaling may play a role in the genesis and functions of renal glomeruli and mesangial cell density in the adult kidney [18]. It has also been shown that the RXFP2 gene is expressed in prostate and thyroid carcinomas, indicating some role in tumor progression [19, 20]. However, due to an absence of well-characterized specific RXFP2 antibodies, the majority of studies focused on RXFP2 gene expression at the RNA level. An anti-human RXFP2 antibody has been reported recently, providing evidence of RXFP2 immunohistologic localization to both Leydig and spermatogenic cells of adult human testis [21].

Here we describe the analysis of different RXFP2-specific antibodies that were used to study mouse RXFP2 expression in different male reproductive and nonreproductive organs, the expression profile of the gene during prenatal and postnatal development, and the effect of INSL3/RXFP2 signaling on gubernacular and Leydig cells. We have also produced a transgenic mouse strain (Rxfp2-iCre) with Cre-recombinase expression controlled by a 2.4-kb Rxfp2 promoter, and we analyzed the effect of different transcription factors on the regulation of Rxfp2/RXFP2 expression.
MATERIALS AND METHODS

Tissue Samples

Mouse tissues were collected from wild-type animals of FVB strain and crsp/crsp mice (complete deletion of Rxfp2) [12] at different stages of development. The PCR genotyping protocols have been described previously [12]. Time-pregnant (E16.5) outbred Wistar rats were obtained from a commercial supplier (Harlan, Indianapolis, IN). All experiments were conducted using the standards for humane care in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine Animal Care and Use Committee.

Quantitative and Conventional RT-PCR

The tissues of three to six wild-type FVB males were used for RNA preparations using an RNeasy Mini kit (Qiagen, Valencia, CA). The cDNA from different organs of prenatal embryos (E16.5; newborn (Day 0); 5-, 10-, and 20-day-old and 8-wk-old males was prepared using RETROscript first-strand synthesis kit (Ambion, Austin, TX). Quantitative RT-PCR (QRT-PCR) for Rxfp2 was performed on RNA samples isolated from different male organs using TaqMan Assays on the Bio-Rad iCycler IQ Real-Time Detection System. The following primers were used in the PCR reaction: GreatF, GreatR (Table 1), and the dual-labeled (FAM-CAATCACTTCCTG GATCGTGGTTTTTTTCC-TAMRA) probe. The amplification of the serial dilutions of full-length Rxfp2 cDNA plasmid was used to build the reference. All data were normalized to Gapdh RNA expression.

The RNA from E15.5 male reproductive tissues was isolated from four Rxfp2-iCre embryos using RNAqueous-Micro kit with DNaseI treatment (Ambion) and converted into cDNA as described above. The expression of Cre recombine, Sox9, and Rxfp2 (primers 4F and 5R) was analyzed using RT-PCR (Table 1). RT-PCR with primers from ubiquitously expressed Hmbs (hydroxymethylbilane synthase) was used to assess the quality of cDNA pools [22]. To control for possible DNA contamination, the corresponding RNAs were used instead of cDNA pools in RT-PCR reactions.
mice were identified by PCR analysis using coding sequences of the localization signal peptide of the simian virus 40 (SV40) large T antigen, and plasmid [27], containing 1.1 kb of a consensus Kozak sequence, the nuclear cccgatccTGCGGCCGGTGTGTGCG-3

Leydig cells. RXFP2 expression was detected in premeiotic ing only in wild-type tissues (Fig. 1), and therefore they were RXFP2 protein. All of the antibodies displayed immunostain-

LS-A4751, and LS-A4754) and two against peptides derived sequence [12], and thus the absence of RXFP2 expression in mutant mice. Of five antibodies tested, three were raised mutant mice. Of five antibodies tested, three were raised sequence [12], and thus the absence of RXFP2 expression in

GAATGTATGTCTACAAAAAG-3

with Sac I and Bam HI and subcloned into SacI/BglII sites of pGL3B-iCre plasmid [27], containing 1.1 kb of a consensus Kozak sequence, the nuclear localization signal peptide of the simian virus 40 (SV40) large T antigen, and coding sequences of the iCre gene [28]. A 3.5-kb SacI/SstI DNA fragment from the iCre expression vector, pGL3B-RXFP2-iCre, was purified from an agarose gel and microinjected into the pronuclei of fertilized eggs of FVB mice. The resulting transgenic mouse lines were named Tg(Rxfp2-cre)Aia or, hereafter, Rxfp2-iCre. Mouse tail DNA was used for genotyping. Rxfp2-iCre mice were identified by PCR analysis using iCre primers (see above). The PCR conditions for genotyping the 205 allele were reported previously [27].

β-Galactosidase Staining

The floxed ROSA, R26R mice were generously provided by Dr. Austin Cooney (Baylor College of Medicine, Houston, TX). Rxfp2-iCre mice were crossed with R26R to generate Rxfp2-iCre, R26R mice. The embryos and adult organs were stained for β-galactosidase according to the previously described protocol [22]. After staining, tissues were fixed, dehydrated, embedded in paraffin, and sectioned. Tissue sections were counterstained with eosin.

Statistical Analysis

Student t-test and ANOVA were used to assess significance of differences among the different groups. P < 0.05 was considered significant.

RESULTS

Characterization of RXFP2-Specific Antibodies

Transfection of embryonic kidney cell line 293T with mouse Rxfp2 DNA expression construct led to the expression of RXFP2 protein, which was detected by RXFP2 antibodies derived from goat and rabbit (Supplemental Fig. 1, available online at http://www.bioreprod.org). Then, we compared the staining of paraffin sections obtained from wild-type and crsp/ crsp mouse testes. The crsp mutation is a 500-kb genomic deletion leading to a complete deletion of the Rxfp2 coding sequence [12], and thus the absence of RXFP2 expression in mutant mice. Of five antibodies tested, three were raised against peptides derived from the extracellular parts (sc-22017, LS-A4751, and LS-A4754) and two against peptides derived from the intracellular parts (LS-A4752 and LS-A4753) of the RXFP2 protein. All of the antibodies displayed immunostaining only in wild-type tissues (Fig. 1), and therefore they were considered to be specific and suitable for RXFP2 expression analysis in mouse tissues. Although the staining pattern of the five antibodies raised to different parts of the receptor varied slightly (Fig. 1), all antibodies detected RXFP2 protein in Leydig cells. RXFP2 expression was detected in premeiotic and postmeiotic germ cells, but not in mature spermatozoa, and only some antibodies (Fig. 1A) produced a signal in Sertoli cells (Fig. 1). By contrast, nonimmune goat or rabbit sera from the same commercial suppliers did not produce any staining in the 293T cells transfected with the mouse Rxfp2 expression construct or in any of the mouse testis sections tested. We were not able to detect RXFP2-specific bands by Western blot in tissue extracts using any of the above antibodies.

Rxfp2 Gene Expression During Development

Previous studies showed that Rxfp2 messenger RNA can be detected as early as Day E7 embryos [13], with the highest expression in adult gubernacular ligaments, testis and, to a lesser extent, in other mouse tissues [16, 22]. Using quantitative RT-PCR, we evaluated Rxfp2 expression in seven different organs in E16.5 male embryos, newborn, 10-day-old, 20-day-old, and adult males (Fig. 2). No Rxfp2 expression was detected in heart, and only weak expression was seen in whole-brain RNA. In most other organs, the expression of Rxfp2 increased with age, with highest levels of expression being detected in gubernacula. Gene targeting experiments indicated that the INSLS3/RXFP2 signaling plays a critical role during the first, transabdominal phase of testicular descent [2, 3, 12, 13]. Interestingly, the expression of Rxfp2 dramatically increased after birth in mice, especially in cremaster muscles, suggesting some role of INSLS3 signaling in this organ.

To evaluate RXFP2 expression at the protein level, we performed immunohistochemistry using specific anti-RXFP2 antibodies and paraffin sections from E14.5 and E16.5 embryos, newborn, 10-day-old, and adult male mice. It was shown previously in embryonic tissues that Rxfp2 is represented by several splice variants recognized as bands of different sizes in Northern blot analysis [13]. To account for this fact and ensure broad coverage of the immunohistochemical detection, we employed three antibodies raised against peptides derived from different parts of the RXFP2 protein. The A4751 and A4754 (second extracellular loop and third cytoplasmic domain of RXFP2) and sc-22017 (ectodomain) antibodies failed to reveal the presence of RXFP2 in prenatal and newborn embryonic sections, indicating low levels of gene expression (Fig. 3, A–C). In 10-day-old mice, RXFP2 expression was detected in gubernacula, testis, epididymis, and kidney (Fig. 3, D–G), and even stronger immunostaining was observed in adult mice (Fig. 3, H–J). Histologic evaluation revealed that the most significant staining was detected in the muscle cords of the cremaster muscle, with less intensive staining in the mesenchymal cells. In the testis, RXFP2-specific immunostaining was observed in Leydig cells, meiotic cells, and especially in postmeiotic germ cells (Fig. 1). In the epididymis, a strong expression of RXFP2 was confined to the columnar epithelium (Fig. 3F). In the kidney, specific RXFP2 expression was confined to the renal distal tubules/collection ducts and was absent in glomeruli (Fig. 3I).

Proliferative Effects of INSLS3 on Gubernacular and Leydig Cells

The cellular growth effect of INSLS3 hormone on primary gubernacular cells and the Leydig cell lines TM3 and mLTC-1 was analyzed using colorimetric proliferation assay. These cells expressed the full-length Rxfp2 receptor, as determined by RT-PCR. Incubation with INSLS3 peptide of gubernacular cells isolated from E16.5, E18.5, and newborn embryos as well as the mouse Leydig tumor cell line mLTC-1 resulted in marginal proliferative response (10%–15%). By contrast, mouse TM3
Leydig cells responded with a dose-dependent increase in proliferation (Fig. 4A), with an almost 40% increase in cell numbers after 48 and 72 h of incubation with hormone (Fig. 4B). Importantly, the TM3 cells displayed a level of Rxfp2 mRNA expression similar to that of the adult mouse testis, whereas the tumor Leydig cell line mLTC-1 showed about three times lower levels of Rxfp2 expression, as evaluated by semiquantitative RT-PCR (Fig. 4C). Similar growth-promoting effect was observed with the androgen receptor agonist methyltrienelone, R1881 (Fig. 4), indicating active androgen signaling in these cells.

Characterization of Rxfp2-iCre Transgenic Mice

We used the rVISTA software package to look for the evolutionary conserved transcription factor-binding sites within promoter regions of the mouse, rat, human, dog, and cow RXFP2 genes [29]. Comparison of the mouse and human nucleotide sequences located 5′ from the translation initiation codon indicated that the conserved promoter region of the gene is limited to the proximal 2.4 kb of the gene sequence. Luciferase reporter constructs driven by a 5-kb mouse Rxfp2 promoter fragment and a 3.4-kb human RXFP2 promoter fragment exhibited significant basal promoter activity in both postnatal mouse and prenatal rat primary gubernacular cells (Fig. 5).

To define the promoter fragment capable of directing embryonic gubernacular expression, we produced Rxfp2-iCre transgenic mice containing the 2.4-kb Rxfp2 promoter region (−2429 bp to 1 bp), 1.1 kb of iCre coding sequence, and an SV40 late poly(A) signal. Microinjection of the transgene (Fig. 6A) into the pronuclei of fertilized eggs of FVB mice followed by transfer of the microinjected eggs into pseudopregnant mothers produced two founder mice (nos. 1 and 5) that harbored the transgene. Both founders transmitted the transgene to their progeny, and two mouse lines were established. The expression analysis of mice from two lines showed similar profiles, and therefore we present here data derived using mouse line 5.

R26R reporter mice were used to determine the expression profile of the Rxfp2-iCre transgene. After intercrossing two strains, we obtained mice with Rxfp2-iCre/+, R26R/+ genotype. Beta-galactosidase staining in tissues collected from such mice indicated that Cre recombinase was able to turn on LacZ gene expression. As expected, blue staining was observed in testes, epididymis, and cremaster muscles of the adult Rxfp2-iCre/+, R26R/+ mice. The β-galactosidase staining was also observed in the developing testis, epididymis, and gubernacular ligaments at E14.5, when the transabdominal phase of testicular descent occurs (Fig. 6B). To confirm the expression of iCre at this time point, we isolated RNA from male embryonic tissues and performed RT-PCR (Fig. 6C). The
expression of both iCre and Rxfp2 was detected in the testis, epididymis, and gubernacular ligament, although only weak expression was observed in the vas deferens. Thus, the 2.4-kb 5' promoter region contains regulatory sequences capable of driving the expression of the Rxfp2 gene in these male reproductive organs during the abdominal phase of testicular descent.

SOX9 Upregulates Promoter Activity of the Human RXFP2 Gene

The male-specific role of the INSL3/RXFP2 ligand-receptor system suggests that the expression of the receptor might be regulated by transcription factors known to be involved in gonadal development. We analyzed the effect of different transcription factors (TFs) on RXFP2/Rxfp2 promoter activity in co-transfection experiments using human embryonic fibroblast 293T cells and primary rat gubernacular cells isolated from E16.5 embryos. The SKY, SF1, SOX9 (Fig. 7A), WT1, HOXA10, MRF2/DESRT, TGIF, DAX1, OCT1, OCT2 (data not shown) were used in co-transfection experiments with RXFP2-luc or Rxfp2-luc constructs. Analysis of luciferase activity revealed that only SOX9 caused a significant increase in human RXFP2 promoter activity in both cell types. In rat embryonic gubernacular cells, SOX9 activated human RXFP2 promoter by more than 20-fold (Fig. 7A). Sox9 expression was readily detectable by RT-PCR in mouse gubernacula at E14.5 (Fig. 7C). However, SOX9 failed to activate mouse Rxfp2 promoter construct transfected in E16.5 rat gubernacular cells (Fig. 7A). Since SOX proteins are known to share the consensus binding sequence [30] with other members of the SOX family of transcription factors, we examine the ability of SOX5 and SOX6 to regulate RXFP2 promoter. The results of these experiments indicated that the transactivation effect was specific for SOX9 protein and not mimicked by SRY, SOX5, or SOX6 (Fig. 7B). Screening for potential SOX9 binding sites within 2.4 kb of human RXFP2 promoter sequence revealed an evolutionary conserved SOX9-binding sequence, AACAAT, located within the distal part of the promoter in human, chimpanzee, and dog, but not in the mouse or rat genome. Despite these findings, even shorter, 1.6-kb RXFP2-luc construct, which does not contain this putative SOX9-binding site, responded to SOX9 co-transfection (Fig. 7B).

It was shown previously that transactivation by the SOX9 transcription factor is dependent upon phosphorylation by protein kinase A (PKA) [31]. To verify this effect, RXFP2 reporter assays were carried out in the presence of adenylate cyclase activator forskolin and the PKA inhibitor H89. Treatment with forskolin or H89 without SOX9 did not cause statistically significant changes in the activity of RXFP2-luc reporter co-transfected with vector DNA. However, 2.5 μM forskolin treatment after co-transfection with SOX9 increased the ability of SOX9 to activate RXFP2 promoter by approximately 2-fold (P < 0.05). Pretreatment with 2.5 μM H89 diminished this effect (Fig. 7C). Thus, the SOX9-
dependent activation of RXFP2 promoter appears to be regulated by cAMP signaling in gubernacular cells.

**DISCUSSION**

RXFP2 is a member of the leucine-rich repeat-containing receptor family, which also includes such well-known receptors as FSHR, LHR, and THR [12, 14]. RXFP2 was shown to be a cognate receptor for insulin-like factor 3 by in vitro and in vivo experiments [14, 32]. Gene targeting experiments demonstrated that the INSL3/RXFP2 pathway is critical for the first trans-abdominal stage of testicular descent in mice, which occurs in mice between E14 and E16 and in humans between 8–15 wk of embryonic development [2, 3, 12, 13]. In addition, the unique T222P mutation in human RXFP2 was shown to be exclusively associated with testicular maldescent [13, 33]. Consistent with these findings, Rsf2 mRNA is present in the embryonic gubernacular ligament [12], confirming the role of RXFP2 in this developmental process. Rsf2 mRNA expression in kidney, brain, bone marrow, and thyroid tissues, as well as in prostate and thyroid carcinoma cell lines, was also noted [12, 14, 18–20, 34, 35], implying that INSL3/RXFP2 signaling may also be linked to nonreproductive functions. However, a positive RT-PCR signal does not necessarily correlate with the presence of protein production. Moreover, it is difficult to determine cell-specific expression within a tissue using this method. In this study, we evaluated several commercially available RXFP2-specific antibodies and used them to assess at the receptor protein level the gene expression data obtained in mouse tissues during different developmental stages. Furthermore, we have generated Rsf2-iCre transgenic mice expressing iCre under the control of the 2.4-kb mouse Rsf2 promoter. The iCre mRNA was detected in the gubernacular ligament at E15.5, indicating that this promoter is able to drive the expression of Rsf2 during transabdominal testis descent. Analysis of Rsf2 promoter-based reporters suggested that in humans, the SOX9 transcription factor may play a specific role in regulating RXFP2 gene activity.

We identified several RXFP2-specific antibodies suitable for immunohistochemistry. Although we were not able to perform antigen competition experiments with peptides used to generate the corresponding antibody, we performed several control experiments to confirm their specificity. Positive staining was detected in 293T cells transfected with mouse Rsf2 cDNA. Significantly, none of these antibodies produced staining in organs isolated from Rsf2-deficient crsp/crsp mice, again indicating their specificity. It was previously reported that Rsf2 reaches maximal expression in the adult testes [17]. Using quantitative RT-PCR, we showed that Rsf2 expression was indeed increased after birth in several organs. Immunoreactive Rsf2 was clearly present in testes and gubernacula of adolescent and adult mice but, surprisingly, it was undetectable in prenatal embryos or newborn mice. Gene targeting studies showed that RXFP2 expression is necessary for the androgen-independent transabdominal stage of testicular descent [12, 13], implying that a certain level of gene expression occurs during embryogenesis. Indeed, the results of in situ analysis showed that in E14.5 embryos Rsf2 expression is present...
exclusively in the gubernacula [16]. Such inconsistency between RNA expression and immunoreactive protein data might be explained by the low level of protein expression in embryonic tissues or by the presence of a protein splice variant undetectable by the available array of antibodies. The latter explanation, however, seems unlikely, since all of the antibodies raised against different parts of the transmembrane domain or the exodomain of RXFP2 failed to produce detectable signals in embryonic tissues. The RXFP2 transmembrane domain is essential for the ability of receptor to transduce the hormonal signal. Thus, receptor splice variants without transmembrane domain will be nonfunctional. We favor the hypothesis that failure of positive staining in embryonic tissues is likely due to a low level of RXFP2 GPCR present in the plasma membrane. Indeed, based on an INSL3 binding assay, a recent estimate of the number of RXFP2 molecules expressed per cell indicated a very low level of endogenous receptors [36]. Nevertheless, these low levels of RXFP2 may suffice to mediate the specific INSL3 signaling in target tissues.

The strongest expression of RXFP2 was detected in adult mouse cremaster muscles. In postnatal mouse gubernacula, RXFP2 protein was first detected in differentiating muscle cells in the base of gubernacular bulb [9]. The high homology of INSL3/RXFP2 ligand/receptor to the closely related relaxin hormone and its receptor suggests that INSL3 signaling similarly might be important for muscle cell function or to promote myogenic differentiation of embryonic fibroblasts at the base of the gubernacular bulb.

In rodent testis, the cell-specific distribution of Rxfp2 has been previously studied by in situ hybridization [37]. In adult rat testis, the signal was localized primarily in germ cells but was absent in Leydig cells. It was argued that the Leydig cells express mainly nonfunctional splice variants of the receptor [21]. Here we show that Leydig cells of the adult mouse testis consistently demonstrated the presence of immunoreactive RXFP2 employing antibodies directed toward different parts of the INSL3 receptor. The expression of RXFP2 in Sertoli cells was detected only with some antibodies, possibly indicating the existence of alternative splicing isoforms. The production of immunoreactive RXFP2 along with the RXFP2 ligand, INSL3, in Leydig cells suggests the existence of autocrine/paracrine signaling within the intertubular testicular compartment and between Leydig and cells of the seminiferous tubules. Of the two mouse Leydig cell lines employed for the study of functional INSL3-responsive RXFP2 signaling pathways, only TM3 Leydig cells responded to INSL3 with increased proliferation. The TM3 cells were originally derived from a prepubertal mouse testis, whereas the mLTC-1 were produced from mouse Leydig tumor. The downregulation of the RXFP2 expression in mLTC-1 resembles a similar downregulation in INSL3 that we described previously in human Leydig cell adenoma [38], and it may contribute to the diminished proliferative response toward INSL3 by these tumor cells. It has been reported that treatment with INSL3 suppresses male germ cell apoptosis induced by a GnRH antagonist in vivo, demonstrating the importance of the INSL3/RXFP2 system in mediating gonadotropin actions [17]. It should be noted that INSL3 signaling only appears to play a supporting role in testis function, since spermatogenesis and fertility can be restored by surgical relocation of cryptorchid testes of Insl3- or Rxfp2-deficient mice [3, 12, 39]. The disruption of INSL3 signaling does not affect Leydig cell differentiation or proliferation, as can be seen in Rxfp2- or Insl3-deficient testis, and the
The last column represents the activity of human 1.6-kb vector (black bars), mouse 5-kb transactivation is specific for SOX9. Relative luciferase activity of pGL3 promoter DNA, RXFP2-luc reporter construct (gray bars) after co-transfection with pCR3.1 vector, RXFP2 promoterless pGL3 vector (black bars) and human 3.5-kb Rxfp2-luc (striped bar) reporter in the presence of SOX9. Neither SRY nor the closely related SOX5 or SOX6 activated human RXFP2 promoter. In accordance with published data [31], we found that treatment of gubernacular cells with agents increasing intracellular cAMP level enhanced SOX9-induced RXFP2 transactivation. Similarly, the PKA inhibitor H89 effectively ablated the forskolin-induced and SOX9-mediated effects on RXFP2 gene activity at a concentration for selective PKA inhibition. Together, these experiments suggest that RXFP2 expression is sensitive to modulations of cAMP-dependent pathways and might be a target for the SOX9-dependent regulation. It was shown previously that cAMP is an intracellular second messenger of RXFP2 receptor, and thus the increased expression of RXFP2 might represent an important autoregulatory activation mechanism. The significance of cAMP for testis descent in the context of analysis of another factor affecting gubernacular differentiation, named calcitonin gene-related peptide, was reported previously [42].

The Sox9 expression was clearly detected in mouse gubernacular ligament at E15.5. Surprisingly, however, no Sox9 activation was detected in co-transfection experiments with the 4.8-kb mouse Rxfp2 promoter reporter, whereas human 3.5-kb and even smaller 1.6-kb promoter fragments responded to SOX9 co-transfection. It is possible that the site responsible for the direct or indirect action of SOX9 on the mouse promoter is located outside the analyzed fragment. It is interesting to note that the campomelic dysplasia caused by SOX9 haploinsufficiency or mutations in humans is often associated with cryptorchidism (OMIM 608160). Analysis of the human, but not mouse, promoter sequence revealed the presence of a potential SOX9 binding site at 2.4 kb within the 5′-prime region of the initiation codon. Deletion analysis failed to confirm the significance of this site for SOX9 activation.
thus indicating that the SOX9 binding might occur through different or multiple sites, or may in fact be indirect and require other transcriptional (co-)factors. It is clear also that other factors are also involved in the activation/suppression of RXFP2 expression in different tissues, as only weak expression of RXFP2 was detected in adult human Sertoli cells [21], which strongly express SOX9. Past studies have identified the crucial role of SOX9 in sex determination [43] and testicular development [44]. Our data suggest that SOX9 plays an important role in testicular descent, and thus is identified as a novel player in this integral part of male reproductive development.

ACKNOWLEDGMENTS

We would like to thank Drs. A. Cooney (Baylor College of Medicine, Houston, TX), V. Lefebvre (The Cleveland Clinic Foundation, Cleveland, OH), K. McElreavey (Pasteur Institute, Paris, France), and R. Sprengel (Max-Planck-Institute for Medical Research, Heidelberg, Germany) for kind gifts of different cDNA expression constructs.

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