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Short Communication

Murine expression and mutation analyses of the prostate androgen-regulated mucin-like protein 1 (*Parm1*) gene, a candidate for human epispadias

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ABSTRACT

Background: Epispadias is the mildest phenotype of the human bladder exstrophy–epispadias complex (BEEC), and presents with varying degrees of severity. This urogenital birth defect results from a disturbance in the septation process, during which separate urogenital and anorectal components are formed through division of the cloaca. This process is reported to be influenced by androgen signaling. The human *PARM1* gene encodes the prostate androgen-regulated mucin-like protein 1, which is expressed in heart, kidney, and placenta.
Methods: We performed whole mount *in situ* hybridization analysis of *Parm1* expression in mouse embryos between gestational days (GD) 9.5 and 12.5, which are equivalent to human gestational weeks 4–6. Since the spatio-temporal localization of *Parm1* corresponded to tissues which are affected in human epispadias, we sequenced *PARM1* in 24 affected patients.
Results: We found *Parm1* specifically expressed in the region of the developing cloaca, the umbilical cord, bladder anlage, and the urethral component of the genital tubercle. Additionally, *Parm1* expression was detected in the muscle progenitor cells of the somites and head mesenchyme. *PARM1* gene analysis revealed no alterations in the coding region of any of the investigated patients.
Conclusions: These findings suggest that *PARM1* does not play a major role in the development of human epispadias. However, we cannot rule out the possibility that a larger sample size would enable detection of rare mutations in this gene.

1. Introduction

Epispadias is the mildest phenotype of the human bladder exstrophy–epispadias complex (BEEC), and presents with varying degrees of severity. Previous research has implicated both environmental and genetic factors in the development of the BEEC.

The non-steroidal androgen receptor antagonist flutamide (Flu; 2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]propanamide), which is used to reduce androgen effects in patients with prostate cancer, has been shown to induce epispadias in male rat offspring, with a peak incidence in animals whose mothers had received a single oral dose on gestational day (GD) 16 (Foster and Harris, 2005). This suggests that reduced androgen levels during early rat development influence the formation of epispadias. In epispadias, migration of the genital tubercle progenitor cells to the cloacal membrane is disrupted, and this provokes failure of the ventral closure of the urethral plate. The opening of the urethra is therefore located on the dorsal part of the penis/clitoris or presents as open groove on the dorsum of the penis.

Few data are available concerning the molecular processes underlying the role of reduced androgen levels in the formation of epispadias. However, we considered the prostate androgen-regulated mucin-like protein 1 (PARM1) a promising candidate, since its regulation is androgen-dependent, and it is involved in cellular responses to environmental stress.

Abbreviations: BEEC, bladder exstrophy–epispadias complex; ER, endoplasmic reticulum; GD, gestational day; PARM1, prostate androgen-regulated mucin-like protein 1; UPR, ER stress/unfolded protein response; WISH, whole mount in situ hybridization.

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The gene encoding Parm1 was first identified in the ventral rat prostate and has been reported to be over-expressed in the regressing prostate following androgen withdrawal (Bruyninx et al., 1999; Fladeby et al., 2008). Previous studies have implicated human PARM1 in cell proliferation and prostatic cell immortalization (Cornet et al., 2003), as well as in murine adipocyte differentiation (Song et al., 2009). Recent research has shown that PARM1 is a widely expressed protein, which is localized to the endoplasmic reticulum (ER), Golgi, and plasma membrane within the early endocytic pathway (Fladeby et al., 2008; Isodono et al., 2010). Here, it is involved in ER stress-induced apoptosis in the rat, which is similar to the function of its yeast ortholog Wsc4 in the cellular response to environmental factors such as oxidative stress and hypoxia (Zu et al., 2001). *Wsc4* expression has been reported to be cell-cycle regulated, with maximum levels occurring at mitosis (Spellman et al., 1998) and, Wsc4 may play a role in protein trafficking (Mamoun et al., 1999). PARM1 may have a similar function, as a previous study demonstrated its involvement in the ER stress/unfolded protein response (UPR) in rat cardiac myocytes, where it silenced expression of *Atf6* and *Perk* (Isodono et al., 2010). The UPR pathway is activated when an accumulation of misfolded and unfolded proteins induces these proteins to aggregate, a process which results in cellular toxicity (Kozutsumi et al., 1988). Signaling cascades induced by the UPR response include the activating transcription factor 6 (ATF6) and the eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3 or PERK) pathways. ATF6 is an ER stress-regulated transmembrane transcription factor, which activates the transcription of the ER chaperone genes that promote correct folding and ER-associated protein degradation. In contrast, PERK couples protein folding in the ER to polypeptide biosynthesis by phosphorylating eIF2-alpha, thereby attenuating the initiation of translation in response to ER stress. Ultimately, sustained ER stress leads to the activation of UPR initiated proapoptotic pathways (Xu et al., 2005).

To our knowledge, no previous study has investigated the involvement of *PARM1* in the early development of the urogenital system. We therefore performed whole mount *in situ* hybridization (WISH) analyses in mice to investigate the expression of *Parm1* between GD 9.5 and 12.5, which corresponds to human gestational weeks 4–6 (O'Rahilly, 1979). This timeframe represents the relevant stages of external genital formation in both species (Wood et al., 2003). Localization of mouse *Parm1* expression was found within the anlage of structures affected in human epispadias. This observation prompted us to perform sequence analysis for mutations in *PARM1* in 24 patients (16 males and 12 females) with isolated epispadias.

2. Materials and methods

2.1. Animals and in situ hybridization

Mouse embryos from wild type NMRI matings were dissected into ice-cold phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde/PBS and then processed for *in situ* hybridization as described by Richter et al. (2004). Digoxigenin (DIG)-labeled antisense RNA probes were transcribed *in vitro* from a polymerase chain reaction (PCR) product of murine *Parm1* (nucleotides 215–523, GenBank accession number: NM_145562) containing a T7 promoter site at the 3′ end. T7 RNA-polymerase, transcription buffer, and nucleotide mix (Promega, Roche, Switzerland) were used in accordance with the manufacturer's recommendations. Probes were purified using G-50 Sephadex columns (GE-Healthcare Giles, UK). Images of whole embryos were obtained using a Leica MZ16A dissecting microscope (Wetzlar, Germany), and processed using Zeiss Axiovision software (Jena, Germany).

2.2. Histological analyses of mouse embryos

Following color development with nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP), embryos were post-fixed in 4% paraformaldehyde and embedded in 20% albumin/13% sucrose/0.5% gelatin/2.5% glutaraldehyde in a PBS matrix. Vibratome sections were prepared with a thickness of 20 μm.

2.3. DNA sequencing

Blood samples were obtained from 24 patients with epispadias (14 males and 10 females) and their relatives, following the provision of written informed consent. All subjects were of self-reported European descent, reported an unremarkable family history, and had a normal karyotype. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn.

2.4. DNA isolation and sequence analysis

Isolation of genomic DNA was carried out using the QIAmp DNA Blood Kit (Qiagen, Hilden, Germany). Analysis of the human *PARM1* gene (located on human chromosome 4q13.3) was performed using PCR. PCR-amplified DNA products were subjected to direct automated sequencing using a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA) in accordance with the manufacturer's specifications. PCR primers also served as sequencing primers. All primer sequences are available upon request. Direct sequencing generated information concerning several single nucleotide polymorphisms (SNPs; dbSNP Build136) in *PARM1*. Nucleotides were numbered according to GenBank entry NM_015393.3.

3. Results

3.1. Analysis of Parm1 expression during midgestational mouse development

Parm1 expression first became evident at the early headfold stage (GD 7.5–8.0). Transcripts were detected within the extraembryonic mesoderm, as well as in the endoderm of the anterior intestinal pocket and the dorsal midline endoderm (Fig. 1, A and A′). At GD 9.0–9.5, *Parm1* expressing cells were found in the cephalic and circumpharyngeal neural crest, the dorsal aspect of the somites, the caudal lateral plate mesoderm, the yolk sac, and in the cloacal membrane

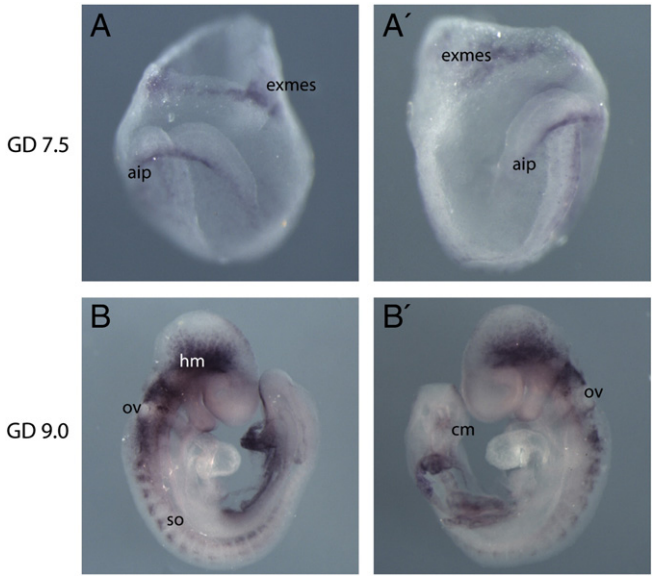

Fig. 1. Lateral views (left, A; right, A′) of a mouse embryo at GD7.5, showing *Parm1* transcripts in the distal part of the anterior intestinal portal (aip) and extraembryonic mesoderm (exmes). (B and B′) left and right views of an embryo at GD9.0 probed for *Parm1* expression. *Parm1* expressing cells were found in the cephalic and circumpharyngeal neural crest (head mesenchyme, hm), the dorsal parts of the somites (so), the caudal lateral plate mesoderm and the yolk sac (ov), and in the cloacal membrane (cm).

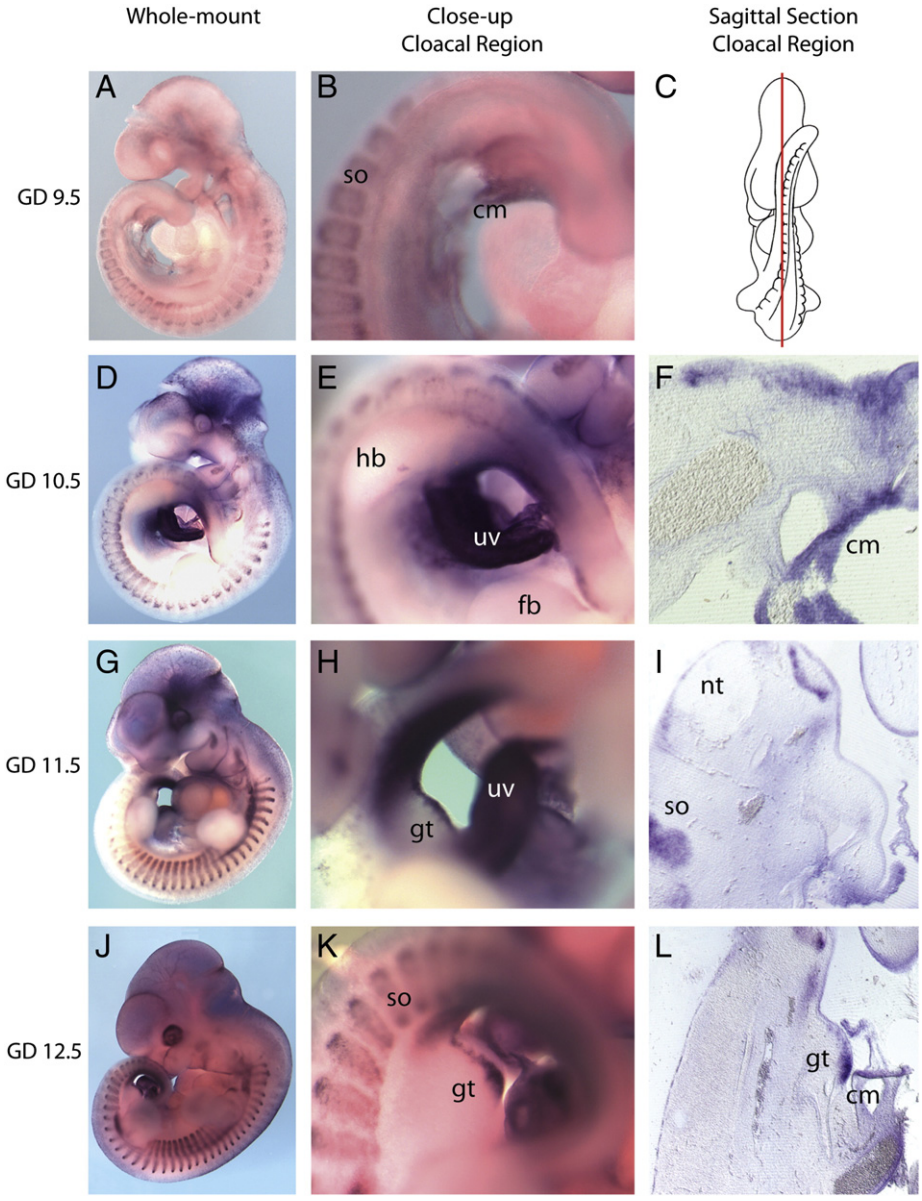

Fig. 2. Expression of *Parm1* during midgestional development of the mouse, as detected by whole-mount *in situ* hybridization. (A) Mouse embryo at GD9.5. *Parm1* transcripts were detected in the somites, cloacal membrane, and head mesenchyme. (B) Close-up of the caudal region of the embryo depicted in (A) showing *Parm1* expression in the cloacal membrane and the somites. (C) Schematic representation of a mouse embryo in frontal view. The red line illustrates the section plane of (F), (I) and (L). (D) Embryo of stage GD10.5 exhibiting strong *Parm1* expression in the cloacal and umbilico-allantoic regions, in addition to the somites and head mesenchyme. The ventrocaudal expression domains are clearly shown in the close-up (E) and the sagittal section (F) of the region. (G) In embryos at GD11.5, *Parm1* transcripts are detected in the progenitor cells of the body musculature and head mesenchyme. (H and I) Close-up and sagittal section of the genital region of the embryo depicted in (G), showing strong *Parm1* expression in the ventral tail mesenchyme, the urethral component of the genital tubercle, and in the umbilico-allantoic region comprising the bladder anlage. (J) *Parm1* expression in an embryo at GD12.5, showing its somitic and cephalic expression domains. (J and K) Close-up (K) and sagittal section (L) of the genital region of the embryo depicted in (J). *Parm1* transcripts are found in the distal aspect of the genital tubercle. Abbreviations: cm, cloacal membrane; fb, forelimb bud; gt, genital tubercle; hb, hindlimb bud; nt, neural tube; so, somite; uv, umbilical vessels.

(Fig. 1, B and B′). These *Parm1* expression domains persisted, and expression became more restricted during mid-gestational development (GD 9.5–10.5, Fig. 2A, B, and D–F). Cephalic and circumpharyngeal expression became focused to the diencephalic head mesenchyme surrounding the eye-primordia, the midbrain–hindbrain region, and the pharyngeal arch mesenchyme, and was detected at these locations until GD 11.5 (Fig. 2G). At GD 12.5, cephalic *Parm1* expression became confined to the eye primordia (Fig. 2J). Within the somites, *Parm1* expression became restricted to the dorsal and ventral lips of the dermomyotome, and persisted in the muscle progenitor cells at all investigated stages (Figs. 2A, D, G, and J). In the ventrocaudal trunk, strong *Parm1* transcriptional activity was detected in the tissues ventral to the hindlimb bud, including the umbilico-allantoic region, the endoderm, and the ectoderm of the cloacal membrane, as well as the ventral ectoderm and mesoderm along the tail including the ventral ectodermal ridge. Thus *Parm1* expressing regions in the caudal embryo during midgestation (GD 9.5–10.5) comprised the progenitors of the urethra, the genital tubercle, and the bladder.

With the emergence and development of the genital bud between GD 11.5 and 12.5, urogenital expression of *Parm1* became focused to the urethral plate endoderm, with a stronger signal being detected in the distal part of the urethral plate (Figs. 2G–L). On the basis of this specific and restricted expression of *Parm1* in the tissues contributing to the morphogenesis of the infraumbilical region – including those of the external genitalia – and its previously established role in androgen-dependent signaling, we hypothesized that PARM1 protein may be implicated in the occurrence of malformations of the external genitalia in humans.

3.2. PARM1 gene analysis in patients with epispadias

Examination of all *PARM1* exons and their adjacent splice sites in the 24 patients revealed only two heterozygous variants. One very rare non-synonymous c.G548A substitution predicting a p.Gly99Ser substitution (dBSNP136: rs14492199) was found in a male patient and his unaffected mother. An as yet unknown c.1101+9C>T substitution in intron 3 was detected in another male patient, whose parents were not available for screening. The following variants, which are common SNPs that have been described previously (dbSNP Build136), were also detected, and the number of the respective alleles is indicated in brackets: rs75120017 (G,46/C,2); rs3822109 (C,26/T,22); rs3775527 (A,23/G,25); and rs1062293 (A,21/G,27). Similar haplotype data were found in the European population of the International HapMap project, the CEPH (Centre d'Etude du Polymorphisme Human) pedigrees, and the PDR90 (The NIH Polymorphism Discovery Resource; 90 individual screening) subset. For some listed SNPs (rs79974259, rs6836354, rs35489484, rs34305542, rs11552358, rs34923973, rs62314892, rs62314897), the nucleotide given in the reference sequence was only detected in the homozygous state.

4. Discussion

Parm1 expression was first detected by Bruyninx et al. (1999) in the ventral rat prostate. The authors demonstrated that *Parm1* showed specific activity in the lower urogenital tract and displayed androgen-regulated expression. In the present study, detailed expression analysis in the mouse embryo focused on the ventrocaudal trunk region during developmental stages that are critical for the growth and patterning of the external genitalia. Spatiotemporal analysis of gene activity in the mouse embryo can provide important clues as to the possible function of a gene in a given developmental process. The results of the present WISH analysis support and extend the findings of Ogaki et al. (2011), who recently detected *Parm1* expression in GD8.5 mouse definitive endoderm. At GD10.5, *Parm1* transcripts were detected in the pericardiac tissues, thus corroborating data from Isodono et al. (2010), who reported on mRNA expression in hearts of mouse embryos at this gestational age.

Our WISH data suggested that *Parm1* is implicated in the development of human epispadias. However, mutational analysis of the gene in 24 epispadias patients identified only two variations in the coding regions or exon-intron boundaries of *PARM1*. In one male patient a p.Gly99Ser variant was observed, which was inherited from his unaffected mother. This rare variant has recently been deposited into the SNP database (rs14492199). Moreover, the program Mutation Taster (www.mutationtaster.org), which evaluates the disease-causing potential of a sequence alteration, predicted that it was a neutral variant. The c.1101+9C>T substitution in intron 3 observed in a second male patient is also likely to be a benign variant. Interference with splice site recognition appears unlikely, since estimation of the consensus value (CV) (Shapiro and Senapathy, 1987) of this potential new splice site yielded a CV of only 0.615, compared with the wildtype CV of 0.911. Given these observations, genomic *PARM1* mutations are unlikely to be a frequent cause of human epispadias. Although the present patient cohort may have been too small to detect rare causal mutational events, the estimated birth prevalence for epispadias is 1 in 117,000 in males and 1 in 484,000 in females (Gearhart, 2002), and thus the sample size can be considered relatively large. Furthermore, we cannot exclude the possibility that we may have missed mutations in either the promoter region (in as yet unknown regulatory sequences) or in non-coding regions which cannot be detected with the method applied. In addition, dysfunction of other factors involved in the regulation of *PARM1* transcription may also lead to differences in its expression. However, no RNA from the patients was available for the performance of such expression analyses. Furthermore, the extent to which such postnatal findings reflect the situation during early embryonic development, when the morphogenetic events leading to epispadias occur, awaits elucidation.

In summary, the present study showed that the *Parm1* expression domain is located in the vicinity of embryonic tissues involved in the development of the urogenital system, and may thus have direct or indirect effects on this process. This observation supported the hypothesis that human PARM1 may be involved in the formation of the defects observed in epispadias and the BEEC. However, our preliminary mutation screening study did not detect *PARM1* mutations in patients with epispadias, although rare mutations might be detectable in larger patient samples. Future studies on the etiology of these malformations should consider genes that encode other proteins in the PARM1 signaling pathway as possible candidates.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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