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New transcription factors involved with postnatal ventral prostate gland development in male Wistar rats during the first week

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ABSTRACT

Aims: The high incidence in men of prostatic diseases, including benign and malignant tumors, makes the understanding of prostate development and biology very important. Understanding the organogenesis of the prostate gland has been a substantial challenge as "prostatic code" is not well defined at the present time. The novelty of this work lies in unveiling new transcription factors (TFs) during neonatal ventral prostate (VP) gland development in male Wistar rats.
Main methods: The techniques of qRT-PCR and immunohistochemistry have been employed to perform this work while the VP gland was obtained from neonatal rats at day zero (the day of birth) day 3 and 6.
Key findings: 16 TFs were studied and we found an increased expression of Eya2, Lhrh and Znf142, invariable levels of Znf703 and Dbp, and decreased expression of 11 others at postnatal development day 3 and 6 as compared to day zero. ZNF703 was found by immunohistochemistry in epithelial cells at days 0, 3 and 6. qRT-PCR for Eya2 and Dmrt2 showed the highest and lowest fold change for them respectively, and immunohistochemistry showed that the former is being expressed at the three selected time points while the latter appears to be diminishing with very few cells expressing it until day 6.
Significance: Results from this work is reporting the role of these TFs for the first time and will significantly contribute to the current understanding of the development and branching morphogenesis of the neonatal VP gland during the first week of postnatal development.

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1. Introduction

The prostate is an exocrine gland which is associated with the urethra immediately below the urinary bladder. During ejaculation, secretions from the many ducts that comprise the gland are discharged into the prostatic urethra by muscular contractions. Male accessory sex glands such as the prostate, seminal vesicle and bulbourethral gland have served in various capacities as models for investigating the action of androgens. The impetus for investigating the regulation of prostatic growth and function stems in part from the many pathological complications, which affect this gland. The prostate is the site of various types of inflammatory and infectious conditions as well as benign and malignant proliferative changes in aging males [1].

In humans, prostate gland development occurs during the second and third trimesters of gestation, whereas in the rodent, a commonly studied model system, budding initiates late in fetal life and branching morphogenesis occurs postnatally [2,3]. At birth, the rodent prostate consists of solid unbranched, elongating ducts extending into the ventral, lateral and dorsal urogenital sinus mesenchyme to form the separate ventral prostate (VP) lateral (LP) and dorsal (DP) prostate lobes with lobe-specific branching patterns [4].

Prostate gland development is an androgen-dependent process with the early events regulated through androgen receptor (AR) in mesenchymal cells [5,6]. As with all branched structures, appendicular patterning and cell differentiation, prostatic development are dictated by common and organ-specific morphoregulatory genes that are expressed in a unique temporal and spatial pattern [6]. Continuous branching morphogenesis of glandular structures is dictated by time-specific and region-specific expression of master regulatory genes. Although common morphogenetic paradigms exist for all branched structures studied to date, the critical difference is that spatial and temporal combinations of these genes give rise to unique structures. The morphogenetic codes for lungs and limbs have been studied extensively and serve as excellent models [7]. While the "prostatic code" is not well defined at the present time, recent activity in this field has led to an early map [8–13].

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AR is expressed in the mesenchyme and induces prostatic epithelial development, which implies that there is an unidentified secreted mesenchymal factor that mediates the action of androgens. After this initial hormone-dependent stage, the development of the prostate is characterized by epithelial–mesenchymal interactions, resulting in cell differentiation and branching morphogenesis, that involve key molecules such as (FGFs, SHH, BMPs, HOXA13 and HOXD13) in addition to a few others (for example, CD44 and follistatin) [14,15]. Since these factors are also expressed to originate other organs, how does the same set of control genes lead to the formation of these distinct structures? This remains unknown, but interacting partners might be expressed differentially in these structures, or different thresholds or temporal and spatial combinations of expression of these key genes might occur in different tissues [16].

Branching morphogenesis of the prostate and seminal vesicles is unique because of the critical role played by endocrine hormones, especially androgens. Many years of descriptive and experimental embryological work have provided substantial insight into the roles of circulating hormones and epithelial–mesenchymal interactions in controlling branching morphogenesis in the prostate and seminal vesicles [14]. In recent years, several specific gene products and genetic pathways have been identified that act at the molecular level to control branching morphogenesis, but these genes do not explain in molecular terms how branching morphogenesis is initiated and patterned. It is likely that additional genes will be identified that play a critical role in regulating branching morphogenesis in the prostate and seminal vesicles [17].

Desai and coworkers published a microarray of the prostate gland in which it was clearly shown that the most prominent effect of androgen is on VP with 1496 genes and ESTs change their expression until one week after castration. The authors pointed out two major trends i.e. 634 genes and ESTs increases their expression in hypoandgrogen environment while another cluster of 690 genes and ESTs decreases their expression post castration. After extensive data mining of the data, intriguingly, we found some TFs that showed transitory expression pattern until one week after castration different than the majority of androgen stimulated or repressed genes and ESTs.

In light of the above findings we selected 16 TFs from the microarray data [18] and further studied them to reveal new group of transcription factors that are involved with neonatal male Wistar rats postnatal prostate gland development during the first week which as previously stated is an important developmental stage of the organ.

2. Materials and methods

Following materials and methods were used to carry out this work:

2.1. Animals

For the postnatal development studies, the VPs of rats killed at days 0 (PND 0; postnatal development day 0) or the day of birth, PND 3 and PND 6 were used. Due to the small size of the prostate gland at these stages, a pool of at least 6 rats in each age was used. All rats were sacrificed by decapitation and the VP was dissected under a stereoscopic microscope. The procedures were approved by the Committee for Ethics in Use of Animals (CEUA) for the Institute of Biology, State University of Campinas protocol no 3000–1.

2.2. Prostate Sample Collection

Eppendorf tubes for the collection of VP samples for RNA extraction were pre-treated with H_2O_2. VP samples for morphology were collected in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The volume of the PFA solution used was approximately 30 times the total volume of the tissue fragments. For RNA and protein extraction the samples were immediately frozen in liquid nitrogen and stored in at −80 °C until used.

2.3. RNA Extraction and Reverse Transcription

VPs were dissected under RNAse free conditions. Subsequently, the tissue fragments were extracted using Illustra RNAspin Mini Kits (GE Healthcare, Germany) according to manufacturer's instructions. RNA purity was analyzed by the absorbance ratio 260/280 (values higher than 1.8) and by electrophoresis in 1.2% agarose gel under denaturing conditions. The RNA concentration in each sample was determined in an Ultraspec 2100pro spectrophotometer (Amersham Biosciences). 5 μg of total RNA was reverse transcribed with 200 U SuperScript III (Invitrogen Corporation) and oligo (dT)12–18 primer (Invitrogen Corporation), according to manufacturer instructions. cDNA was quantified by spectrophotometry.

2.4. Designing of Primers and Probes

Primers and probes for the selected genes were designed using the Gene Runner 3.05 program and confirmed by BLAST search. Details are given in Table 1.

2.5. Quantitative RT-PCR

Quantitative real-time polymerase chain reaction (qRT–PCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in the Applied Biosystems 7300. Inventoried assays (Primer and FAM-conjugated probes) mentioned in Table 1, were purchased from Applied Biosystems. cDNA (20 ng) was used in each reaction, according to universal cycling conditions for the TaqMan system. The results were normalized using the CT (threshold cycle) values of the internal control beta-2 microglobulin (B2m) on the same plates. B2m was chosen as internal control because it was found to show the least standard deviation among the experimental groups among 7 others tested for this purpose as shown in Table 2 with all the details including CT and standard deviation. The equation $\Delta CT = CT$ (target gene) – CT (internal control) was employed for normalization of the results. In order to quantify and acquire the fold-change variation of our genes the mathematical model $2^{-\Delta\Delta CT}$ was utilized. Our genes and B2m assays had their efficiency calculated through the equation: $E = 10^{(-1/\text{slope})}$. All reactions were performed in technical triplicate on the same plate for each pool, and the experiment was repeated twice.

2.6. Immunohistochemistry

The VPs of animals at PND 0, 3 and 6 were collected and immersed in Tissue-Tek O.C.T. Compound (Torrance, CA, USA) and frozen in liquid nitrogen. Five-μm cryosections were obtained and used for immunofluorescence. Sections were fixed in cold methanol followed by 2% paraformaldehyde for 10 min each. The sections were permeabilized with 0.2% Tween 20 in PBS for 15 min at room temperature. The autofluorescence was quenched with 10% H_2O_2 in PBS for 15 min. Non-specific protein-protein interactions were blocked by incubation with 10% preimmune serum in PBS for 1 h at room temperature. The sections were incubated overnight with the antibodies from ABCAM (Cambridge, MA, USA) ZNF703 (diluted 1:100), EYA2 and DMRT2 antibodies (diluted 1:300). In these experiments the tissue-bound primary antibody was detected with a 546 Alexafluor conjugated goat anti-rabbit Ig (Invitrogen Carlsbad, CA, USA). The sections were visualized in Zeiss LSM810 confocal microscope using the same parameters for all studied samples. Moreover, all the data were analyzed in triplicate for reproducibility of the results.

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