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Regional localization of activin-β_A, activin-β_C, follistatin, proliferation, and apoptosis in adult and developing mouse prostate ducts

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ABSTRACT

Activins and inhibins, members of the TGF-β superfamily, are growth and differentiation factors involved in the regulation of several biological processes, including reproduction, development, and fertility. Previous studies have shown that the activin-β_A subunit plays a pivotal role in prostate development. Activin-A inhibits branching morphogenesis in the developing prostate, and its expression is associated with increased apoptosis in the adult prostate. Follistatin, a structurally unrelated protein to activins, is an antagonist of activin-A. A balance between endogenous activin-A and follistatin is required to maintain prostatic branching morphogenesis. Deregulation of this balance leads to branching inhibition or excessive branching and increased maturation of the stroma surrounding the differentiating epithelial ducts. Recent work identified another member of the TGF-β superfamily, the activin-β_C subunit, as a novel antagonist of activin-A. Over-expression of activin-C (β_C-β_C) alters prostate homeostasis, by interfering with the activin-A signaling. The current study characterized the spatiotemporal localization of activin-A, activin-C and follistatin in the adult and developing mouse prostate using immunohistochemical analysis. Results showed activin-C and follistatin are differentially expressed during prostate development and suggested that the antagonistic property of follistatin is secondary to the action of activin-C. In conclusion, the present study provides evidence to support a role of activin-C in prostate development and provides new insights in the spatiotemporal localization of activins and their antagonists during mouse prostate development.

1. Introduction

The prostate is an exocrine gland that functions as part of male reproductive tract in mammals. The mouse model has emerged as a useful model for studying the prostate development due to the similarities with the human prostate development (Powers and Marker, 2013). For example, altought the prostate morphology varies widely among species, the epithelial branching morphogenesis is a key feature for its development in most mammals including rodents and humans (Thomson and Marker, 2006). Our knowledge on the mechanism that controls prostatic and seminal vesicle branching morphogenesis derives from experimental work conducted in the embryological field as well as from the study o mice and humans harboring mutation that alter branching morphogenesis (Thomson and Marker, 2006).

In mice, the result of ductal branching and morphogenesis leads to a multi-lobed organ which comprises anterior (AP), ventral (VP), dorsal (DP) and lateral (LP) prostate. Within each lobe the entire ducts can be divided into three segments: proximal, intermediate and distal; this classification is based on the relative distance of the three segments from the urethra and it is also based on the variation in cellular morphology. For example, the adult distal region is characterized by epithelial cells with a high proliferative activity; these cells are columnar, tall and produce secretory proteins. Evidence of apoptosis is rarely observed in the adult distal region. The proximal region on the other hand, demonstrates high apoptotic activity and the epithelial cells characterizing this region are low-columnar and cuboidal (Sugimura et al., 1986a; Cunha et al., 1987). Similar regional variation in morphological and functional activities of the prostate were also described in the prostatic ductal system in rats (Lee et al., 1990; Hayashi et al., 1991).

The prenatal development of the prostate is dependent on

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androgens, particularly on DHT (dihydrotestosterone). In fact, treatment of rodents with inhibitors for the 5alpha-reductase (an enzyme necessary for the conversion of Testoterone to DHT) from days 14–22 of gestation impaired the development of the prostate and virilization of the external genitalia in male offspring compared to those in control animals (George and Peterson, 1988).

Tsujimura and co-workers demonstrated that the proximal region of the murine prostatic ducts contains a population of cells exhibiting features of stem cells, with a high proliferative potential capable of reconstituting branched glandular ductal structures from a single cells (Tsujimura et al., 2002). The transforming growth factor Beta (TGF-β) maintains dormancy of the prostatic stem cells in the proximal region of ducts (Salm et al., 2005) and therefore, it has been identified as a crucial molecule for the normal prostatic homeostasis.

Activins are members of the TGF-β superfamily, and are homo (β_A:β_A, β_B:β_B)- or heterodimers (β_A:β_B) of the β_A or β_B subunits linked by a disulphide bridge. Activin-A (β_A-β_A) inhibits basal and androgen-stimulated proliferation and induces apoptosis in the human prostatic cancer cell line, LNCaP (Wang et al., 1996). Additionally, activin-A inhibits growth in prostate tumour cell lines (McPherson et al., 1999).

After their initial discovery, another subset of activin-β subunits (β_C, β_D, β_E) were identified, based on the homology to the β_A and β_B subunits. It has been shown that mice bearing a functional deletion of the activin-β_C and/or -β_E subunit genes did not show developmental defects and were phenotypically normal (Lau et al., 2000). This evidence was used to conclude that activin-β_C is not biologically relevant. In 2009, our group proposed for the first time the hypothesis that in the context of the null mouse, there may be functional redundancy with other transforming growth factor-β family members and that over-expression rather than under-expression is more likely to have physiological consequences. To test this hypothesis, mice over-expressing activin-β_C were produced, and the resulting biological effects were analyzed in testis, liver, and prostate. Human prostate cell lines (LNCaP) were also used to test the *in vitro* effect of activin-β_C (Gold et al., 2009). The study showed that activin-C antagonized activin-A *in vitro.* Specifically, activin-C antagonized the growth inhibitory effect of activin-A in LNCaP cells. Additionally, reduced activation of the intracellular effectors activated by the activin-A signaling pathway was described when activin-β_C subunit was over-expressed both *in vivo* and *in vitro*. The study also showed for the first time that over-expression of activin-β_C *in vivo* alters testis, liver and prostate tissue homeostasis, by interfering with activin-A signaling. Other recent studies conducted by our group suggested that activin-β_C subunit is a significant regulator of activin-A bioactivity, placing it in the same domain as well-characterized regulators such as follistatin (Gold et al., 2009, 2013; Marino et al., 2013, 2014, 2015a, 2015b, 2015c).

Follistatin is not structurally related to activins; it binds to the β-subunits of activin to prevent the interaction with the activin receptors type II (Shimonaka et al., 1991; Nakamura et al., 1990). It has been previously shown that a balance between endogenous activin and follistatin is required to maintain prostatic branching morphogenesis (Cancilla et al., 2001). An excess of activin inhibits ductal branching and inhibition of the epithelium, promotes stromal maturation. On the other hand, an excess of follistatin promotes more branching and increases maturation of the stroma surrounding the differentiating epithelial ducts (Cancilla et al., 2001).

The current study aimed to characterize the spatio-temporal localization of activin-β_A, activin-β_C, and follistatin in the adult and developing mouse prostate using immunohistochemical analysis. Specifically, we wanted to determine if expression of the activin-β_A subunit was expressed in association with increased cell death and whether the antagonists (activin-β_C or follistatin) were expressed in regions with high proliferative activity.

2. Material and methods

2.1. Experimental animals and tissue collection

All experiments were approved by the Animal Ethics Committee of Monash Medical Centre, Clayton, Australia and carried out in accordance with National Health and Medical Research Council guidelines for the Care and Use of Laboratory Animal Act. All animals were housed under 12:12-h light-dark cycle, food and water were available *ad libitum.* Mice on C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the Monash Medical Centre.

Animals were anaesthetized and euthanized by cervical dislocation. Prostate lobes were removed from 5 male mice for each time point (7, 18, 28, 42, 90 days). Using a dissecting microscope, ductal spreads were prepared and performed, prostate lobes were orientated proximal to distal and fixed in Bouin's solution for 4–6 h. Tissues were washed in 70% ethanol, embedded in Paraffin and sectioned at 5 μm onto Superfrost Microscope Slides (Menzel-Glaser).

2.2. Immunohistochemical analysis

Immunolocalization of activin-β_A, activin-β_C, follistatin, PCNA, cleaved caspase-3, high molecular weight cytokeratin CKHMW and actin alpha-smooth muscle (sm-actin) was detected using the DAKO Autostainer Universal Staining System (DAKO A/S, Denmark). The antibodies used were: activin-β_A (abcam cat# 56057), activin-β_C (abcam cat# 73904), follistatin (H-110 Santa Cruz Biotechnology cat# 30194), Proliferating Cell Nuclear Antigen (PCNA) mouse monoclonal antibody (DAKO cat #M0879), caspase-3 (Asp175, Cell Signaling Technology cat# 9661), CKHMW (abcam cat# ab776) and actin alpha-smooth muscle (Sigma-Aldrich cat # A2547).

Immunohistochemistry was performed after microwave antigen retrieval (1000 Watts for 14 min) in buffers as follows: activin-β_A, CKHMW, sm-actin, and PCNA (0.01M citrate buffer pH 6.0), follistatin, activin-β_C (0.01M glycine buffer pH4.5), caspase-3 (0.01M sodium citrate buffer pH 6.0). After cooling, slides were washed three times in PBS, and endogenous peroxidase activity was quenched by using the Peroxidase-Blocking solution (DAKO Real cat# S2023). Sections were treated with CAS blocking reagent (Invitrogen cat# 00-8120) and antibodies incubated as follows: activin-βA, activin-βC and follistatin (90 min at room temperature), PCNA (30 min at room temperature), cleaved caspase-3, CKHMW, sm-actin (2 h at room temperature). Secondary antibodies used were: Polyclonal Goat Anti-Rabbit Immunoglobulin/Biotinylated DAKO cat #E0432, Polyclonal Goat Anti-Mouse Immunoglobulin/Biotinylated DAKO cat #E0433. Negative controls included secondary antibodies only (Supplemental materials). Antibodies were detected with avidin, biotin complex (ABC), for 15 min at room temperature and then color reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAKO Cytomation, DAKO Australia Pty Ltd NSW Australia) for 5 min. Reactions were stopped in water, and sections counterstained with Mayer's Haematoxylin, dehydrated and mounted as previously described (Gold et al., 2004).

2.3. Stereological analysis

The incidence of proliferation (PCNA positive) and apoptosis (caspase-3 positive) in tissue sections was estimated based on a method that allowed an unbiased semi-quantitation of the percentage of positive cells. Tissue sections were mapped at ×10