Histol Histopathol (2014) 29: 815-830
DOI: 10.14670/HH-29.815

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

A role of junction-mediated interactions in cells of the male reproductive tract: Impact of prenatal, neonatal, and prepubertal exposure to anti-androgens on adult reproduction

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Summary. Male sexual development and male reproductive functions are dependent on the normal action of androgens, and an unbalanced ratio of the active androgens can lead to varying degrees of structural and functional abnormalities within the reproductive organs. Endocrine balance can be disturbed by environmental and pharmaceutical anti-androgens (i.e. vinclozolin, phthalates, procymidone, and flutamide) that antagonize normal androgen action. Such chemical compounds enter the cell, bind to the receptor and inactivate transcription leading to disruption of androgen-mediated signaling. Assembling and functioning of cell junctions in hormone-dependent tissues, such as testis, epididymis and prostate appeared to be controlled by steroid hormones, predominantly by androgens. This review presents recent findings on the tight junction proteins mainly responsible for normal functioning of the barrier within the testis, epididymis and prostate, anchoring junction proteins that play a crucial role in normal cell-cell adhesion, and gap junction proteins through which intercellular communication takes place in the male reproductive tract. The review gives examples of animal models that are used in endocrine disruption studies with a focus on the author's own data from studies in the pig.

Key words: Intercellular junction proteins, Testis, Epididymis, Prostate, Flutamide, Boar

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Introduction

Male reproductive health is dependent on normal testicular function and the coordinated release of hormones in the hypothalamic-pituitary-testis axis. Mammalian testis exhibits two main functions: production of steroid hormones (steroidogenesis) and production of germ cells (spermatogenesis). Both processes are controlled by gonadotropins. Therefore, adequate levels of circulating luteinizing hormone (LH) and follicle stimulating hormone (FSH) are essential for biosynthesis of gonadal steroids, the initiation and maintenance of seminiferous epithelium, and normal germ cell development (Dohle et al., 2003). Moreover, normal functions of the epididymis, a major component of the male excurrent duct system, and of the prostate, an important male accessory gland, are of vital interest in adult male reproduction. After completing structural maturation in testis, spermatozoa proceed into the rete testis and then to efferent ductules, the initial segment of the epididymis. For the potency of sperm to fertilize eggs within the female reproductive tract important substances are produced by the prostate (Ekman, 2000).The regulation of epididymal and prostatic epithelial cell functions and their effects on sperm maturation, storage and creation of microenvironment for the sperm are androgen-dependent (Robaire and Viger, 1995; Hernandez et al., 2007). In the past decade a putative role of intercellular junction proteins that form blood-testis-, blood- epididymis-, and blood-prostate barriers (BTB, BEB, BPB) has been demonstrated for the completion of spermatogenesis and for maintenance of homeostasis within epididymal and prostatic

epithelium, respectively (Wong and Cheng, 2005; Cyr et al., 2007; Yan et al., 2008b; Meng et al., 2011; Mital et al., 2011).

Anti-androgens and disorders of male sexual development

A great deal of interest has been focused on decline of male reproductive function regarding potential adverse effects of various chemical compounds, so called "reprotoxicants", that could mimic or antagonize the effects of steroid hormones, like androgens and estrogens (Massaad et al., 2002; Luccio-Camelo and Prins, 2011). It was reported that disruption in testosterone production could alter sperm count, as was observed in the case of banana-farmers using a pesticide with anti-androgenic activity in tropical countries (Wesseling et al., 1997). An official term "endocrine disruptors" (EDs) as exogenous chemical substances that alter the function of the endocrine system and cause adverse effects to an organism has been introduced over the past 20 years (Skakkebaek et al., 2001). The hypothesis has been formulated that one of the major causes of reproductive system disorders could result from hormonal disruption during differentiation and development of the reproductive system (Skakkebaek et al., 2001; Uzumcu et al., 2004; Aucharek et al., 2010). Importantly, the definition of EDs relates to a mechanism of action for a variety of chemicals in humans, domestic animals and wildlife (Edwards et al., 2006; Del Mazo et al., 2013). Such chemical compounds are not readily biodegradable, and they have the potential for bioaccumulation. Thus, endocrine disruption became the main focus of biomedical research and first the compounds with estrogenic activity were searched. According to vom Saal et al. (1997) exposure of rat dams to low doses of estrogenic chemicals, bisphenol A or diethylstilbestrol, during pregnancy affected the growth of accessory glands, including the prostate, in the offspring. Chronic administration of high doses of 4-tert-octylphenol to male rats and bank voles resulted in reduction of the sizes, weights, and histological structures of the testes, epididymides, ventral prostate glands, and seminal vesicles (Boockfor and Blake 1997; Hejmej et al. 2011; Kotula-Balak et al. 2011). However, in recent years it has appeared that "reprotoxicants" with anti-androgenic activity are more relevant for disorders of male reproductive development (Gray et al., 2006; Anahara et al., 2008). Since the testis is the major site for testosterone biosynthesis which plays a crucial role in initiation and maintenance of spermatogenesis, any reduction in testosterone action due to exposure to anti-androgens have the potential to adversely affect human and animal reproduction. Indeed, numerous epidemiological and experimental data indicate that fetal exposure to a variety of compounds interfering with hormonal signaling induce a wide range of male reproductive system abnormalities (for review see Diamanti-Kandarakis et al., 2009). These abnormalities are often manifested at birth, such as hypospadias or cryptorchidism, although in many cases they are not revealed until adulthood, e.g. low sperm count, testicular cancer and prostatitis. Ten years ago Omezzine et al. (2003) found that *in utero* exposure to an antiandrogen flutamide induced in the adult rat testes a chronic apoptotic germ cell death process. Moreover, Anway et al. (2005) demonstrated decreased spermatogenic capacity and increased incidence of male infertility in males of F1 to F4 generations that were exposed during the period of embryonic sex determination to flutamide, suggesting than susceptibility to diseases can be heritable and, in consequence, transgenerationally transmitted through the male germ-line. Administration of vinlozolin to pregnant rats resulted in abnormalities of androgen-regulated sexual differentiation in male offspring, including reduced anogenital distance, nipple retention, hypospadias, cryptorchidism, and decreased growth of sex accessory glands at adulthood (Cowin et al., 2010), whereas exposure to vinclozolin during puberty or adulthood caused a delay in the appearance of the male sexual characteristics. Other studies by the group of Gray (Rider et al., 2008, 2009) revealed that the mixture of chemicals that alter the androgen signaling pathway disrupted male rat reproductive tract differentiation and induced malformations in a cumulative, dose-additive manner, although the chemicals in the mixture did not all share a common cellular and molecular mechanism of toxicity. Further studies in our and other laboratories demonstrated that transient hormonal signaling imbalance in specific periods of fetal and neonatal life has a long-term effect on multiple aspects of reproductive system functioning in adult males. These observations led to set up a concept of a fetal/perinatal origin of adult testicular and prostate disease (Anway et al., 2008; Benbrahim-Tallaa et al., 2008; Kopera et al., 2011; Lydka et al., 2011; Hejmej et al., 2012, 2013). Endocrine disruption is therefore of relevance both in human and experimental animal toxicology.

Androgen-mediated signaling pathway – an anti-androgen action

Androgens play an important role in testicular development and functions, exerting their genomic effects *via* a specific receptor, the androgen receptor (AR) that belongs to the nuclear receptors superfamily. The hormones have the ability to up- or down-regulate their own receptors. The AR is found in all male reproductive organs, it binds on a specific sequence to the gene promoter, called androgen response element (Fig. 1A). Anti-androgens bind to AR and block its function, i.e. flutamide (a pure, non-steroidal anti-androgen) inhibits AR transcriptional activity (Ashby et al., 2002). Changes in AR activity as well as in AR mRNA or protein expression result in altered androgen signaling, which may induce disturbances in androgen-dependent processes later in life (Fig. 1B). The effects

may be exerted through altered gene expression at any stage of development or through changes in organ structure or physiology (Rhind et al., 2003). On the other hand, anti-androgens may not completely block AR signaling and they may exert effects that are not associated with androgen-mediated signaling pathway (Ing, 2005; Anway and Skinner, 2008).

In this review we are not aiming to summarize the numerous studies linked to the role of endocrine disruptors in etiology of reproductive disorders, but instead we focus on the effects of hormonal signaling disruption during pre- and postnatal development on intercellular junctions in reproductive tissues, with special emphasis on the role of anti-androgens. It is not surprising that assembling and functioning of cell junctions in hormone-dependent tissues, such as testis, epididymis and prostate appeared to be controlled by steroid hormones, predominantly by androgens.

Tight junctions in the testis, epididymis, and prostate. Animal models in anti-androgen studies

Tight junctions form the continuous intercellular barrier between epithelial cells, which restrict or control the passage of molecules across the epithelium. In testis and epididymis TJ is the primary component of blood-testis barrier (BTB) and blood-epididymal barrier (BEB), respectively. These barriers are critical for the maintenance of composition of the luminal environment in seminiferous tubules and epididymal duct that is responsible for sperm production and maturation as well as for the protection of spermatozoa from the immune system (Cyr et al., 2007). The blood-testis barrier is unique among other blood-tissue barriers. First, TJ in the seminiferous epithelium is present at the basal region and coexists with other cell junction types, in contrast to other barriers e.g., BEB, where it is located at the most apical region (Franca et al., 2012). Second, BTB is a highly dynamic structure, which undergoes restructuring to enable the entry of preleptotene spermatocytes into the adluminal compartment (Cheng and Mruk, 2002; Yan et al., 2008a). Importantly, recent studies provided evidence that regulation of BTB dynamics is mediated by testosterone and cytokines. Testosterone stimulates TJ proteins transcytosis and recycling, promoting the assembly of TJ fibrils below migrating spermatocytes before cytokines (TGF-ß2, TGF-ß3 and TNFα) induce the disassembly of TJ fibrils above spermatocytes *via* junction proteins internalization and degradation (Meng et al., 2005; Wang et al., 2006; Yan et al., 2008b; Su et al., 2010; Lydka et al., 2012a).

Several TJ transmembrane proteins have been identified between Sertoli cells, e.g. occludin, claudins-1, -3, -4, -5, -7, -8, -11, junction adhesion molecules A and B (JAM-A and JAM-B) and coxsackievirus-adenovirus receptor (CAR). These transmembrane

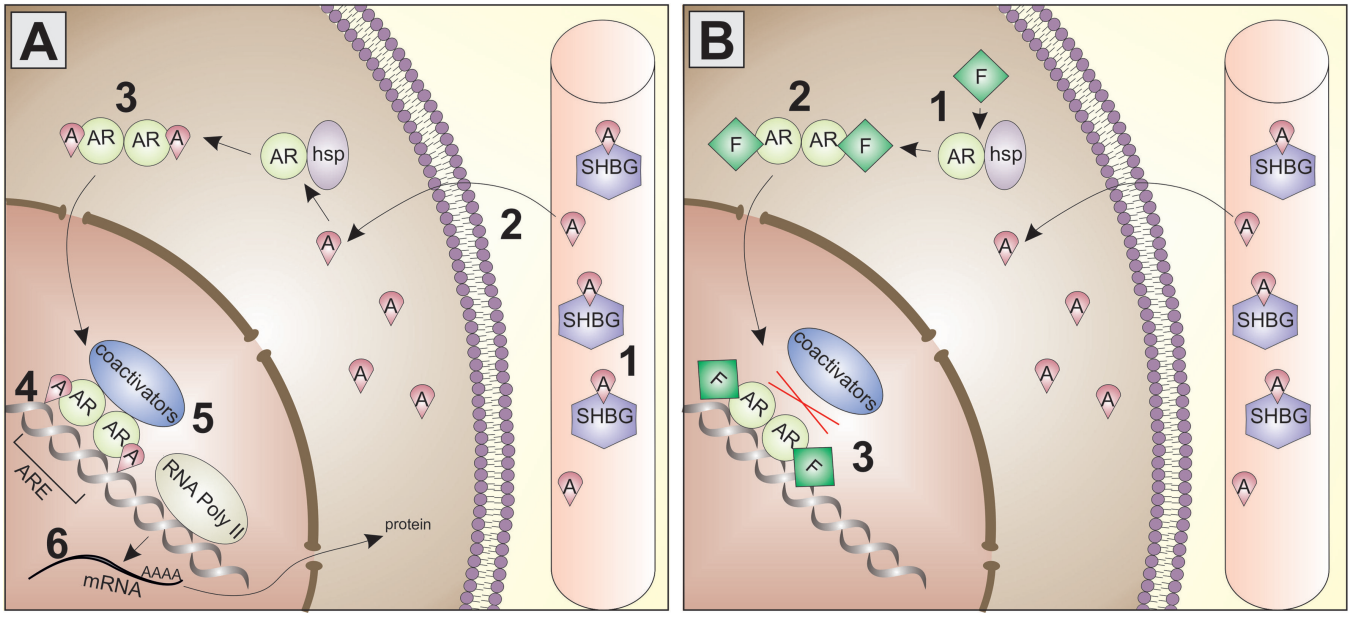

Fig. 1. Schematic representation of androgen action. **A.** The classical androgen signaling pathway. In circulation androgens **(A)** are bound with sex hormone binding proteins (SHBG) (1). Only free androgens (2) enter the target cell by passive diffusion and (3) bind with their cognate receptors (ARs). The AR conformation allows it to be released from heat shock proteins (hsp) in the cytoplasm. After dimerization the hormone-receptor complex is able to translocate to the nucleus and (4) binds to a specific DNA sequence, called androgen responsive element (ARE), on the promoter of genes responsive to androgens. AR binding to an ARE (5) allows the recruitment of co-activators. The binding of the hormone-receptor complex and co-activators (6) activate transcription that leads to alterations of cellular function. **B.** The mechanism of flutamide (F) action. Flutamide as the AR antagonist (1) acts on the target cell level, (2) competitively binds to AR. Blocking the AR (3) inhibits coactivator recruitment and transcriptional activity.

proteins interact with components of the cytoplasmic plaque, such as zonula occludens (ZO-1, -2, -3), cingulin, afadin, or partitioning defective-3 and 6 (PAR-3 and PAR-6), creating protein complexes that control paracellular permeability, gene expression, cell proliferation and polarity (Mruk and Cheng, 2010).

The role of androgens and the androgen receptor signaling pathway in the control of TJ protein expression was demonstrated in adult and prepubertal rats both *in vivo* and *in vitro* (Gye and Oshako, 2003; Kaitu'u-Lino et al., 2007; Su et al., 2010). Studies using a flutamide model revealed that activation of AR is involved in the regulation of occludin and claudin-11 expression in Sertoli cells (Gye and Oshako, 2003; Kait'u-Lino et al., 2007). Furthermore, recent experiments in *hpg* mice demonstrated that androgens promote the localization of claudins at Sertoli cell contacts and TJ formation (McCabe et al., 2012).

Androgen action during pre- and postnatal development also appeared to be important for BTB functioning in adult males. Florin et al. (2005) found that *in utero* exposure to flutamide may induce hypospermatogenesis through alterations of claudin-11 expression before puberty and at adulthood. In contrary, we demonstrated that neither embryonic nor neonatal exposure to flutamide affected occludin expression in the testes of adult boar (Hejmej et al., 2012). However, in damaged seminiferous tubules, found in the testes of adult boars treated with flutamide during the period of sex determination and during neonatal development, reduced expression level and altered distribution pattern of ZO-1 was detected. In normal tubules, immunostaining for ZO-1 appeared as a ring at the BTB, surrounding the entire seminiferous tubule, whereas perpendicular to the basement membrane, a discontinuous pattern of ZO-1 staining was noticed in the affected seminiferous tubules after flutamide exposure (Hejmej et al., 2012) (Fig. 2). Decreased mRNA and protein levels, as well as mislocalization of ZO-1 could be related to the loss of the functional integrity of the BTB, as evidenced previously by both lanthanum tracer study and immunofluorescence (Fink et al., 2006). Thus, it seems evident that spermatogenic impairment observed in animals after transient androgen signaling disruption during development results from BTB dysfunction. Since it was shown that ZO-1 is involved in AJ formation, another implication of altered ZO-1 expression may be disturbed assembly of these

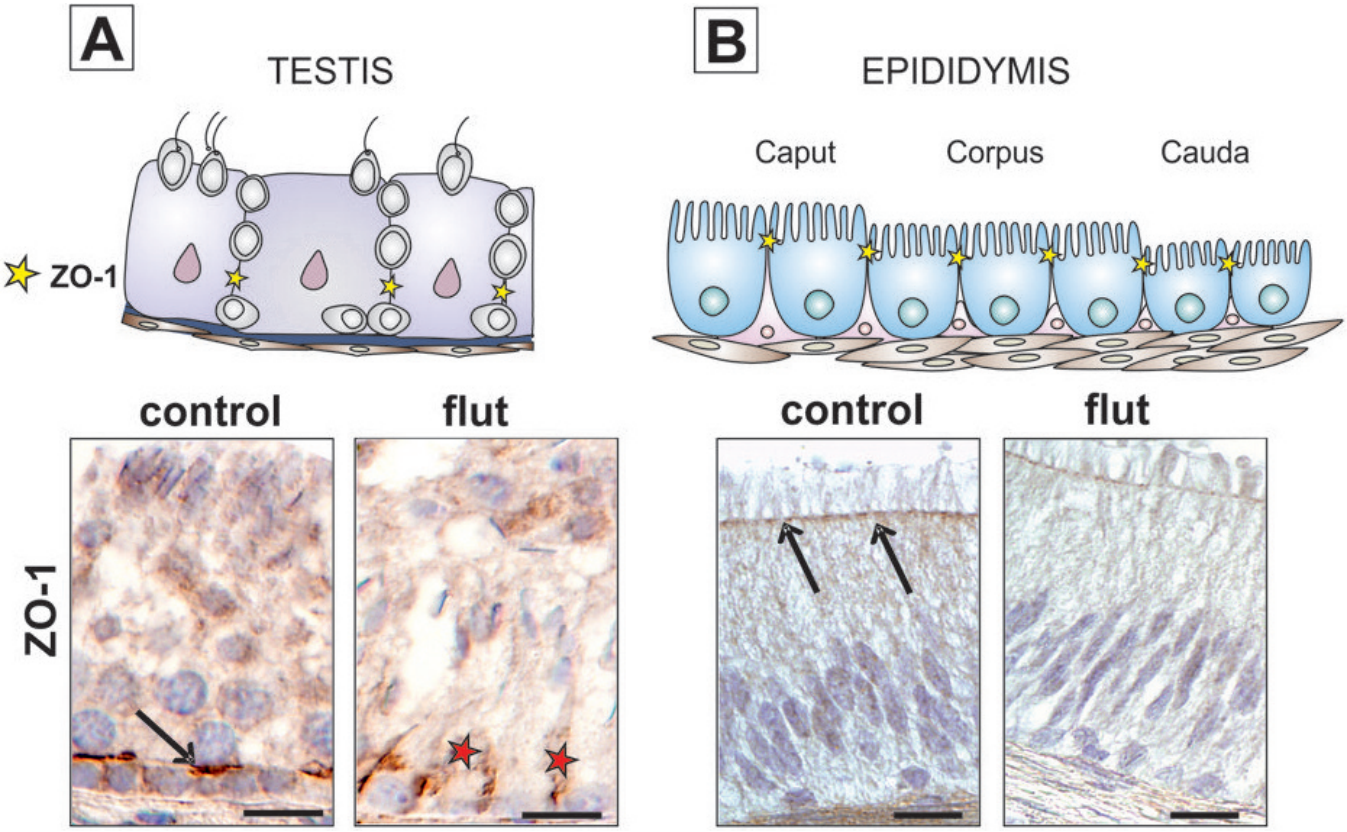

Fig. 2. Schematic diagram of testicular **(A)** and epididymal cells **(B)** in which ZO-1 is localized. Representative photomicrographs of adult boar testis **(A)** and corpus epididymis **(B)** depicting ZO-1 localization in control and flutamide-treated (flut) males neonatally. ZO-1 immunoreaction – arrows. Counterstaining with Mayer's hematoxylin. Protein delocalization – red asterisks. Scale bars: 20 μm.

junctions in flutamide-exposed boars (Ikenouchi et al., 2007).

Phthalates, environmental reprotoxicants which exhibit both antiandrogenic and androgenic activity, were also reported to alter the expression of BTB proteins (Shen et al., 2009). Di(2-ethylhexyl) phthalate (DEHP) affected the expression and distribution of ZO-1 and caused delocalization of claudin-11 in prepubertal rat testis (Sobarzo et al., 2009). These changes appeared to be reversible, although a delay of spermatogenesis was observed in phthalate-treated rats. Expression of BTB proteins were also changed in cultured rat and murine Sertoli cells exposed to testicular metabolite of DEHP, mono-(2-ethylhexyl) phthalate (Yao et al., 2010; Chiba et al., 2012).

As mentioned above, TJ forming BEB occupies the apical compartment of the epididymal epithelium. The number and protein composition of these junctions varies along the epididymal duct, being most extensive in the caput epididymis and reduced in the cauda epididymis (Suzuki and Nagano, 1978a,b; Cyr et al., 2007). In contrast to BTB, which in rodents, domestic animals and human is established at puberty (Setchell et al., 1988), the timing of BEB formation is still controversial; some authors postulate that BEB can be formed in early postnatal life, whereas according to others it takes place during fetal development (Cyr et al., 1999; DeBellefeuille et al., 2003; Guan et al., 2005). Indeed occludin, the first TJ protein identified in the epididymis, was demonstrated in the apical cell surface of the mouse epididymis at gestational day 18.5. Interestingly, membranous localization of occludin coincides with peak androgen levels, indicating that androgens control epididymal TJ formation (Cyr et al., 1999). Peripheral membrane protein ZO-1 associating with occludin and claudins is detected in rat epididymis from the first week of postnatal life (DeBellefeuille et al., 2003). In immature rats it colocalizes with adherens junction protein ß-catenin along the apical and lateral plasma membranes of the epithelial cells, whereas in adult males it is seen apically between adjacent principal cells of the caput, corpus and cauda epididymis. Similar distribution of this protein in the area of apically located TJ was found in the epithelium of adult boar epididymis (our unpublished data). In addition, ZO-1 was detected in myoid cells surrounding epididymal duct of the boar, supporting earlier observations that this protein is also present in non-epithelial tissues (Howarth et al., 1992; Itoh et al., 1993; Laing et al., 2005).

Regulation of epididymal TJ is still poorly understood. Early studies on orchidectomized mice revealed a decrease in the number of TJ in the epididymis, suggesting that their maintenance is dependent on some testicular factors (Suzuki and Nagano, 1976). It was also established that a decrease in the androgen level in aging rats is related to the down regulation of occludin expression and loss of BEB (Levy and Robaire, 1999). Further, Cyr et al. (2007) reported that orchidectomy of adult rats resulted in loss of claudin-1 from BEB in the initial segment of the epididymis, but testosterone supplementation maintained expression of this protein between principal cells. These data indicate that androgens are likely one of the factors controlling TJ formation and function in epididymis. To further elucidate the role of androgen signaling in the regulation of epididymal TJ, we examined ZO-1 expression and distribution in the epididymides of adult boars treated during neonatal and prepubertal development with flutamide. We demonstrated that in adult boars transient exposure to flutamide during both periods led to a decrease of ZO-1 mRNA and protein expression and loss of immunostaining in the TJ area of caput and corpus, but not of the cauda epididymis, indicating that ZO-1 is regulated in a segment-specific manner (unpublished data) (Fig. 1B). It cannot be excluded that reduced ZO-1 is a secondary result of altered responsiveness of principal cells to androgens, since our previous observations reveled that AR expression was markedly decreased in the epididymis of the boar (Lydka et al., 2011).

Over 10 years ago a concept of blood-prostate barrier (BPB) was proposed by Fulmer and Turner (2000). It has been suggested that the blood-prostate barrier is located in the prostatic glandular epithelium, and, similarly to BTB and BEB, prevents molecules from blood and stromal compartment from crossing the glandular epithelium to enter into the prostatic glands lumen. Further studies indicate also the possibility that prostate is an immune privileged organ (Gittes and McCullough, 1974). The hypothesis of BPB is supported by the observation that drug distribution in the prostate and its delivery into the prostatic glands is affected by prostatic epithelium permeability (Wientjes et al., 2005). Moreover, it was demonstrated that epithelial prostatic cells express a wide range of TJ proteins: occludin, claudins, JAM-A, ZO-1 (Danielpour, 1999; Raschperger et al., 2006; Sakai et al., 2007). Interestingly, some of the claudins (claudin-3, -4, -5, -8 -10) present junctional localization in luminal epithelial cells of the prostate gland, characteristic for TJ complexes, whereas others (claudin-1 and -7) exhibit basolateral distribution, suggesting their role additionally in cell–cell and cell–matrix adhesion or signaling (Van Itallie and Anderson, 2004; Raschperger et al., 2006; Sakai et al., 2007). Claudins might also be involved in neoplastic transformation and metastasis, since deregulation of their expression was observed in prostate adenocarcinoma (Long et al., 2001; Morin, 2005).

The role of androgens in the maintenance of prostatic TJ ultrastructure and function has been recently demonstrated by Meng et al. (2011). Using both experimental animals and clinical biopsies the authors determined that the autoimmune response of prostatic tissue associated with low testosterone conditions results directly from compromised BPB function. It was established that loss of immune privilege was related to decreased expression of claudin-4 and claudin-8 mRNA and protein. *In vitro* studies on prostatic cell lines

showed that claudin-7 is also regulated by androgens, whereas expression of occludin seems to be androgen-independent (Zheng et al., 2003; Mitra et al., 2006).

Anchoring junctions in the testis, epididymis, and prostate. Animal models in anti-androgen studies

Anchoring junctions support tissue integrity by linking cytoskeletal proteins of neighboring cells (desmosomes/desmosome-like junctions and adherens junctions (AJ)) as well as cellular proteins to proteins in the extracellular matrix (hemidesmosomes). In the testis both testis-specific AJ, namely basal ectoplasmic specializations (ES), and desmosome-like junctions are located at the site of BTB between adjacent Sertoli cells. Basal ES are composed of integral membrane proteins, type I cadherins (N-, E- cadherins) and nectin, interacting with cytoplsmic proteins, catenins (i.e. α-, ß- and γ-catenins) and afadin, which are linked to actin cytoskeleton (Goossens and van Roy, 2005). Desmosome-like junctions are located not only between neighboring Sertoli cells, but also between Sertoli and germ cells. In desmosome-like junctions desmosomal cadherins (desmocollin, desmoglein) form complexes with proteins of the plakin family (desmoplakin) and the armadillo-repeat proteins (plakoglobin and plakophilins) using vimentin as their attachment site. Interestingly, desmosome-like junctions are replaced by apical ES as differentiation of round spermatids into elongated spermatids follows (stages VII/IX in rat) (Kopera et al., 2010a). In the apical ES located in the adluminal compartment at Sertoli cell-elongated spermatid interface, cadherin/catenin and nectin/afadin complexes are found, in addition to its specific protein complex laminin 333- α6ß1-integrin (Lee et al., 2003; Cheng et al., 2010). Another testis-specific structure, which coexists with the apical ES in the elongated spermatid and replaces it in the last few days before sperm release from the semniferous epithelium is the tubulobulbar complex (TBC). Tubulobulbar complexes are cytoplasmic evaginations of elongated spermatids, comprising a core of F-actin, actin-associated proteins (e.g., vinculin, cortactin, espin), adhesion molecules and double-membrane vesicles composed of clathrin. Tubulobulbar complexes have a primary role in establishing close, but transient contact between Sertoli cells and spermatids before they are released, in removing of excess spermatid cytoplasm and in recycling of junctional molecules (Upadhyay et al., 2012). The latest studies demonstrated that TBCs are also present at the BTB region, where they may be involved in internalization of basal junction complexes (Du et al., 2013).

To test whether cell-cell adhesion in porcine testicular cells could be changed following anti-androgen treatment, exposure to flutamide during two critical windows, prenatal and neonatal, was performed (Hejmej et al. 2012). In neonatally treated pigs altered distribution of N-cadherin and ß-catenin within seminiferous tubules was demonstrated in comparison with its normal localization in the basal and apical ES (Fig. 3A). Moreover, the expression of both adherens proteins was significantly reduced. Such down-regulation of N-cadherin and ß-catenin proteins was also demonstrated in Sertoli cells *in vitro* after exposure to different anti-androgens: flutamide, dieldrin, and MEHP (Fiorini et al., 2004; Chen et al., 2008; Zhang et al., 2008). In contrast, in the study by Sobarzo et al. (2006) DEHP caused an increase in N-cadherin and ß-catenin immunoexpression in the rat seminiferous tubules. In both animal models (rat, boar) deregulation of N-cadherin and ß-catenin expression positively correlated with severity of testicular pathology characterized by the increase in apoptotic cell death frequency and germ cell sloughing which indicated impaired cell-cell adhesion within the tubules. Premature detachment of spermatogenic cells induced by suppression of androgen action during development could be due to altered expression or function of anchoring junction proteins (Sobarzo et al., 2006; Hejmej et al., 2012).The group of Cheng (Xia et al., 2005; Zhang et al., 2005) demonstrated dissociating N-cadherin from ß-catenin at the apical and basal ES in the rat seminiferous tubules using an androgen suppression model. In another study, Anahara et al. (2006) reported adverse effects of flutamide on spermatogenesis in mouse testes. After flutamide treatment they found a stage-specific decrease in the level of cortactin in seminiferous tubules at stages VI-IX that induces deletion of the apical ES and leads to deformation of the spermatid heads.

The structure of anchoring junctions in the epididymis is much less explored when compared with the testis. Electron microscopy studies conducted in the 80's and 90's of 20th century revealed the presence of desmosomes located in the apical region of the epithelium (Greenberg and Forssmann, 1983; Nakai and Nasu, 1991; Cyr et al., 1995). However, to date molecular composition of epididymal desmosomes has been poorly studied (Dube et al., 2010).

Significantly more research has been focused on AJ. In rodent and human epididymis E- and P-cadherin were identified. Their primary function in the epididymal duct is mediating epithelial cell adhesion as well as signal transduction pathways. E-cadherin seems to be the main epididymal cadherin, since in rat its expression reaches peak at puberty and is detected in all regions of epididymis in adult male. The cytoplasmic domain of cadherins forms a complex with several proteins, including catenins. Expressions of α-, ß- and p120 catenins in rat epididymis are region-dependent (DeBellefeuille et al., 2003). For example, the highest expression of α-catenin and ß-catenin was detected in the corpus region, whereas p120 catenin appeared to be prominent in all epididymal regions. In contrast, our studies indicate that in adult boar epididymis there are no evident differences in the immunoexpression of E-cadherin and ß-catenin among epididymal regions (Gorowska et al., 2014). Neonatal and prepubertal

treatment with flutamide down-regulated E-cadherin and ß-catenin gene expression in adult boar epididymis. Evident alterations in the gene expressions were produced after neonatal administration of flutamide (Fig. 3B). A clear decrease in E-cadherin mRNA and protein was demonstrated in the corpus and cauda epididymis (similarly to Cx43 expression, see, a section below), suggesting specific requirements for the junction proteins along porcine epididymis. A decrease in the E-cadherin expression in adult epididymis would result in a decrease in ß-catenin binding to cadherin as reported in the epididymis of orchidectomized rats (Cyr et al., 1992). In another study by the group of Cyr (1993) it was found that the expression of E-cadherin is androgen-dependent in all segments of rat epididymis, since the authors observed a marked increase in E-cadherin mRNA during BEB formation. However, no obvious signs of suppression of androgen action such as failure of development of epididymis, was observed in the rat by the time of birth or early in postnatal life. Also, postnatal exposure to flutamide did not result in testicular maldescent nor failure of development of rat epididymis (Kassim et al., 1997). It should be added that the effects of various toxicants on the efferent ducts and epididymis have been reported by Hess (1998).

Most studies on anchoring junctions in prostate and prostatic cell lines focused on the role of adhesion molecules in tumor progression and metastasis. Among desmosomal proteins identified in prostatic cells, plakophilins are considered as a potential marker of cancer aggressivity (Breuninger et al., 2010). Both E- and N-cadherin are expressed in prostatic epithelium. E-cadherin is mostly expressed in epithelial cells, whereas N-cadherin is present in various cell types, including mesenchymal cells. An increase in N-cadherin expression together with loss of E-cadherin expression is observed during epithelial to mesenchymal transition, as well as in prostate cancer (Jaggi et al., 2006). An important role in prostate carcinogenesis is also attributed to α-catenin, which in some conditions may

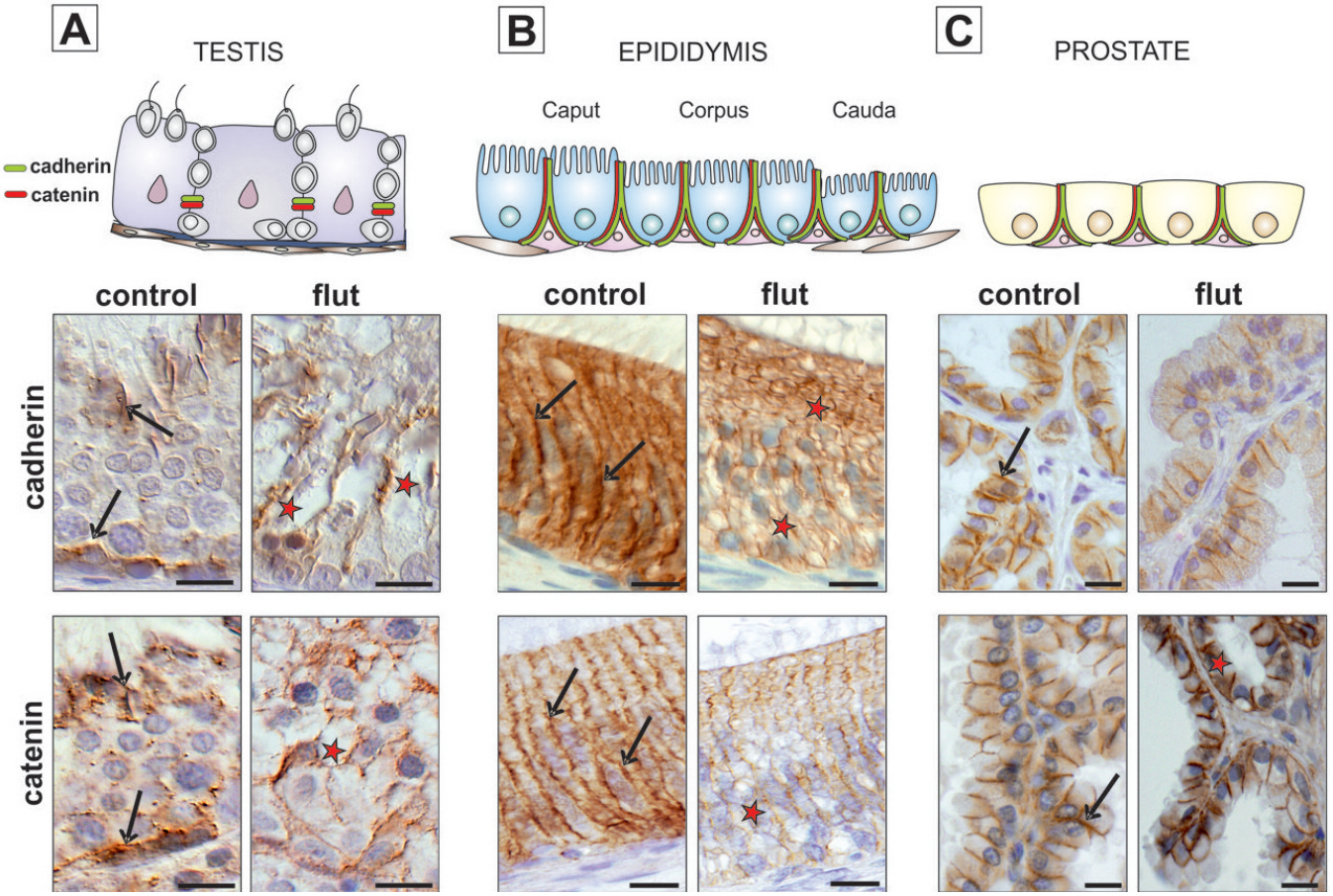

Fig. 3. Schematic diagram of testicular **(A)**, epididymal **(B)** and prostatic cells **(C)** in which N-cadherin **(A)** or E-cadherin **(B, C)** and ß-catenin **(A, B, C)** are localized. Cadherins – red, ß-catenin – green. Representative photomicrographs of adult boar testis, corpus epididymis, and prostate depicting N-cadherin **(A)** or E-cadherin **(B, C)** and ß-catenin **(A, B, C)** localization in control and flutamide-treated (flut) males neonatally. Cadherin and ß-catenin immunoreaction – arrows. Counterstaining with Mayer's hematoxylin. Protein delocalization – red asterisks. Scale bars: 20 μm.

activate expression of genes involved in prostate cancer development (Yardy and Brewster, 2005). In adult boar prostate flutamide administered neonatally caused distinct alterations in E-cadherin and ß-catenin gene expression, although both genes were differentially regulated: E-cadherin expression was down-regulated while the expression of ß-catenin was up-regulated (Gorowska et al., 2014) (Fig. 3C). In both, the epididymis and prostate, deregulation of E-cadherin and ß-catenin gene expressions was probably caused by impaired androgen/estrogen ratio, since significant alterations in the expression of testosterone metabolizing enzymes were noted following flutamide exposure (Gorowska et al., 2014). Over the past 10 years, Rowlands and co-workers (2000) have reported an involvement of androgens in the regulation of E-cadherin and ß-catenin expression in prostate cell lines.

Gap junctions in the testis, epididymis, and prostate. Animal models in anti-androgen studies

The third type of intercellular junction, extensively studied in the male reproductive system, is the gap junction, mediating direct intercellular communication. Gap junctions are formed by homomeric or heteromeric hemichannels, called connexons, localized in plasma membranes of neighbouring cells. Connexons assemble to form intercellular pores, which allow the passage of small molecules (<1kDa) between the cells. Each connexon is composed of six protein subunits - connexins (Cxs). Permeability of connexons is regulated by the phosphorylation state of connexins as well as by intracellular pH, whereas their selectivity is determined by Cx type (Herve, 2007; Pointis et al., 2010). To date about 20 different connexins have been identified in mammals and trancripts for at least 11 Cxs have been detected in rodent testis (Risley, 2000; Söhl and Willecke, 2004).

In the testis Cx43 presence was described in germ, Sertoli and Leydig cells. In early stages of postnatal development in seminiferous cords Cx43 is localized mainly at the apical border of Sertoli cells (Bravo-Moreno et al., 2001; Durlej et al., 2011). As migration of germ cells from the periphery to the center of the seminiferous tubules follows, Cx43 distribution in Sertoli cells changes from apical to basal (Bravo-Moreno et al., 2001). In seminiferous tubules of adult human, rodents and domestic animals (boar, stallion) Cx43, as the most abundant Cx, is localized predomitantly in the basal compartment of seminiferous epithelium between adjacent Sertoli cells at the region of BTB, although it is also expressed in the apical compartment between Sertoli and germ cells and its distribution pattern seems to be stage-dependent (Risley et al., 1992; Pelletier, 1995; Batias et al., 1999, 2000; Steger et al., 1999; Decrouy et al., 2004; Hejmej et al., 2007; Kopera et al., 2011). Recent studies demonstrated that Cx43 colocalizes with TJ proteins, such as occludin, ZO-1 and ZO-2 , and ZO-1 is involved in the regulation of gap junction communication, while Cx43 modulates TJ functions (Carette et al., 2010). Connexin 43 may also form complexes with AJ protein, N-cadherin (Wei

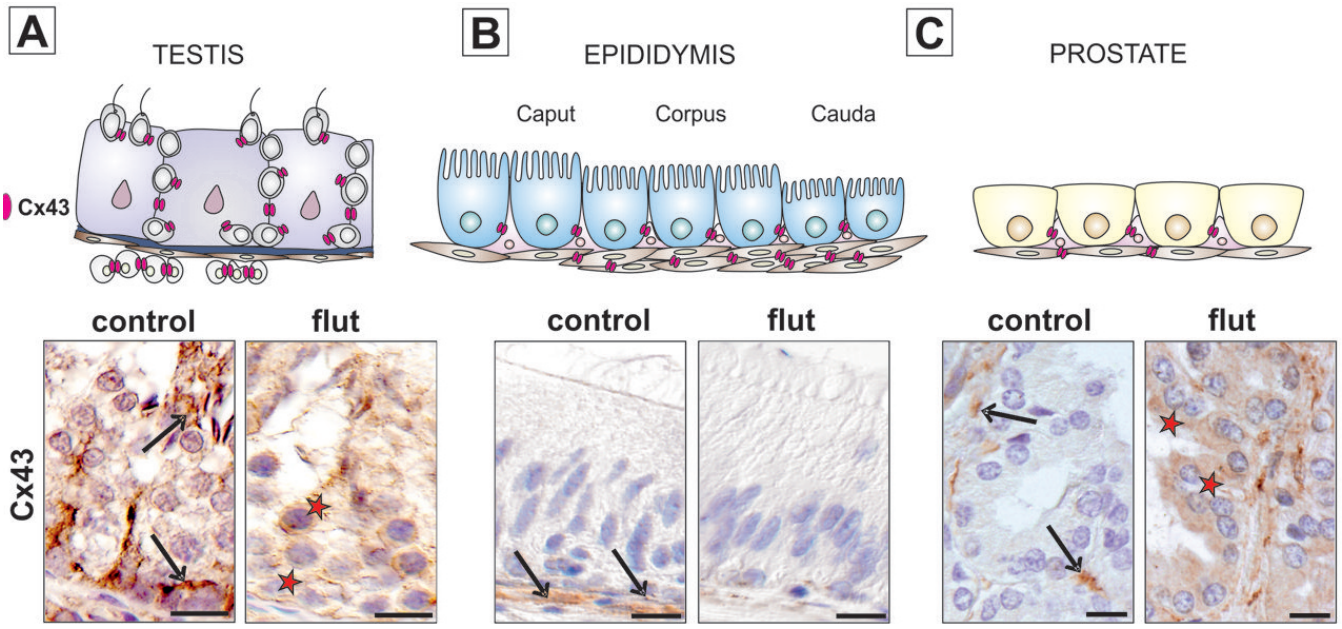

Fig. 4. Schematic diagram of testicular **(A),** epididymal **(B)** and prostatic cells **(C)** in which Cx43 is localized. Representative photomicrographs of adult boar testis **(A)**, corpus epididymis **(B)**, and prostate **(C)** depicting Cx43 localization in control and flutamide-treated (flut) males neonatally. Cx43 immunoreaction – arrows. Counterstaining with Mayer's hematoxylin. Protein delocalization – red asterisks. Scale bars: 20 μm.

et al., 2005). A crucial role of Cx43 in spermatogenesis was demonstrated using Cx43-knockout mice. Because total disruption of the Cx43 gene leads to perinatal death, conditional Cx43 knockout mice, lacking the Cx43 gene solely in Sertoli cells, were generated. In these mice spermatogenesis was arrested at the level of spermatogonia or Sertoli cell-only syndrome was observed (Brehm et al., 2007; Sridharan et al. 2007). Moreover, we found that these effects were accompanied by a partial loss of androgen receptor protein expression, indicating that Cx43 affects the AR signaling pathway in the male gonad (Chojnacka et al., 2012). Surprisingly, Cx43 in germ cells seems to be not essential in germ-Sertoli cell communication, and may be compensated by the persistence of other Cxs (Günther et al., 2013). Connexons composed of two other Cxs, Cx26 and Cx32, were also detected in the seminiferous epithelium (Risley et al., 1992). In contrast to Cx43, both Cx26 and Cx32 are localized mainly in the apical compartment of the epithelium, but their specific function within the testis is still not well understood. In Leydig cells Cx43 is so far the only identified connexin, although dye transfer experiments on Cx43-deficient Leydig cells indicated that Cx43 is not the only protein contributing to their gap junctions (Kahiri et al., 2006). Connexin 43 expression in Leydig cells is maintained from birth until adulthood, but some fluctuations in its mRNA and protein levels are observed throughout the postnatal development (Bravo-Moreno et al., 2001).

Our previous research revealed no influence of prenatal treatment with flutamide on Cx43 expression in testicular cells of neonatal piglets (Durlej et al., 2011) and an unremarkable decrease in the expression of Cx43 in the gonad of prepubertal boars (Kopera et al., 2010b). It was interesting to determine whether androgen withdrawal during critical developmental windows could alter Cx43 gene expression and induce delayed effects on morphology and function of adult pig testes. Indeed, prenatal and neonatal treatment with flutamide caused down-regulation of Cx43 expression within the seminiferous tubule epithelium of adult boar. Moreover, delocalization of ZO-1, N-cadherin and ß-catenin in neonatally exposed pigs was related to altered expression of Cx43 protein, which suggest that androgen withdrawal during neonatal period is critical for proper BTB function later in life of the boar (Fig. 4A). This

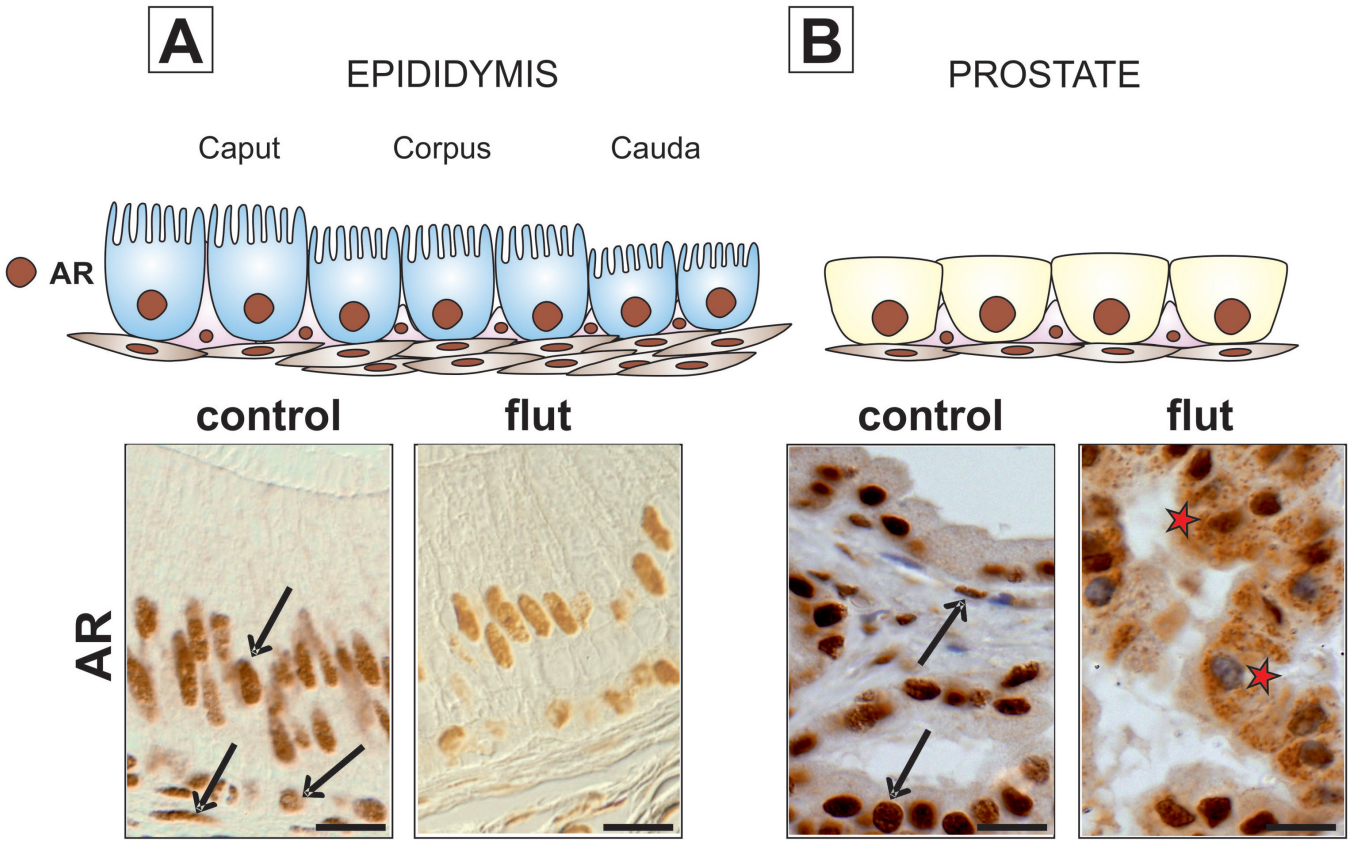

Fig. 5. Schematic diagram of epididymal **(A)** and prostatic cells **(B)** in which AR is localized. Representative photomicrographs of adult boar corpus epididymis **(A)** and prostate **(B)** depicting AR localization in control and flutamide-treated (flut) males neonatally. AR immunoreaction – arrows. Counterstaining with Mayer's hematoxylin. Protein delocalization – red asterisks. Scale bars: 20 μm.

information is relevant for further understanding the role of anti-androgens in male reproduction. Varying degrees of tubule abnormality were observed in testes of flutamide-exposed boars (Kopera et al., 2011), ranging from completely normal tubules with full spermatogenesis to severely abnormal tubules as described previously in the rat testes (Kassim et al., 1997). Concomitantly, the most severely damaged tubules displayed the most decreased level of Cx43. As demonstrated *in vitro*, Cx43 is required not only in cell-cell communication between Sertoli and germ cells, but also in the regulation of other junction proteins essential for the BTB. Strong evidence for this statement comes from studies on mice lacking connexin 43 in which function of TJ and AJ was compromised leading to reduced fertility or even infertility (Carette et al., 2010).

Connexin 43 is also the first identified and to date most studied connexin in the epididymis (Cyr et al., 1996). Studies showed that in rat Cx43 mRNA levels in the caput-corpus epididymis increase until puberty, and then rapidly decrease, while Cx43 mRNA levels in the cauda epididymis do not change markedly during postnatal development (Dufresne et al., 2003). Along the epididymal duct of adult male Cx43 protein is detected predominantly at the basal region of the epithelium between principal and basal cells, whereas no or very scarce signal is observed between adjacent principal cells or between principal and clear cells, although precise distribution of Cx43 is dependent on the region of epididymis. In cauda region this protein is abundant between myoid cells surrounding epididymal duct and may be involved in expelling spermatozoa from the cauda epididymis during ejaculation (Cyr et al., 1996; Dufresne et al., 2003). A similar pattern of Cx43 distribution in the epididymis was observed in different mammalian species, such as rodents, stallion, and boar, suggesting that the role of Cx43 in this organ is conserved between species (Cyr et al., 1996; Hejmej et al., 2007; Cyr, 2011; Lydka et al., 2011). As in adult porcine testes, neonatal administration of flutamide induced the region-specific alterations in the adult epididymis morphology and a distinct decrease in Cx43 expression, mainly in the stromal cells of the corpus and cauda epididymis (Lydka et al., 2011) (Fig. 4B). Clearly reduced AR expression in all the three segments of adult boar epididymis indicated altered AR signaling after flutamide treatment (Fig. 5A). Based on the above results flutamide was suggested as targeting Cx43 protein and affecting the process of epididymal microenvironment formation later in adult life. Consistently, Toyama et al. (2001) and Atanassowa et al. (2005) reported that neonatal administration of hormonally active toxicants induces reductions in testis weight, sperm count and motility in adult rats and generates prominent alterations in the vas deferens and cauda epididymis in the rat at adulthood. Significantly reduced sperm count and motility, although without evident alterations in the morphology of the epididymis, were demonstrated in transgenic mice with a mutation in one of the extracellular loops of Cx43 (Gregory et al., 2011). This can indicate a role of epididymal Cx43 in the sperm maturation process. Recently, it was reported that sperm morphology and sperm functional parameters are impaired as a consequence of *in vivo* and *in vitro* anti-androgen action (Lydka et al., 2012b). Besides Cx43, several other connexins were detected in rat epididymis. In immature males Cx26 is present between adjacent epithelial cells that line the lumen of the epididymis. In the corpus and cauda region when differentiation occurs, they switch from producing Cx26 to Cx32. Thus, in adult male Cx32 is the main connexin mediating communication between adjacent principal cells. In cauda epididymis Cx26 expression pattern is maintained during adulthood, although its level is reduced (Dufresne et al., 2003).

Connexins 43, 32 and 26 are also expressed in the prostate. Interestingly, the expression pattern of Cx43 and Cx32 during development of rat prostate resembles that described for the epididymis. Immunohistochemical analyses showed that during early postnatal development Cx43 expression is strong both in mesenchymal and undifferentiated epithelial cells. As prostatic tissue differentiates, the proportion of Cx43 decreases, whereas Cx32 increases. Finally, in the adult prostate the expression of Cx32 is found in the secretory epithelial cells, and Cx43 is restricted to basal cells, similarly as is seen in caput and corpus epididymis (Habermann et al., 2001, 2002). It is thus possible that changes in connexins composition modulate intracellular messages associated with cellular differentiation of epididymis and prostate. Connexin 26 is almost exclusively expressed in secretory epithelial cells, although, in contrast to the epididymis, it is abundantly expressed in adult prostate (Bijnsdorp et al., 2012). Multiple studies suggest that loss of connexins, especially Cx43 and Cx26, is an important event in prostate cancer formation and development (for review see Czyz et al., 2012).

Our latest results revealed that disruption of androgen signaling by flutamide affects prostatic cell function in adult boars by down-regulation of the AR and Cx43 gene expressions and changes in proliferation and apoptosis rates (Hejmej et al., 2013) (Fig. 4C). In an earlier study Cuhna et al. (2004) reported that cell activity is directly regulated by AR activation in the prostatic epithelium. Reduced expression of AR and its delocalization to the cytoplasm instead of nucleus in some epithelial cells of prostatic acini was evident after neonatal exposure to flutamide (Fig. 5B). Such delocalization of the nuclear receptor protein indicates receptor degradation and/or reduced bioavailability of testosterone (Coffey and Robson, 2012). Using a rat model Huynh et al. (2001) demonstrated that the expression of Cx43 is directly regulated by testosterone in adult males. After orchidectomy they found up-regulation of Cx43 mRNA and protein in the prostate. A second piece of evidence for hormonal regulation of Cx43 expression comes from testosterone replacement, which caused the Cx43 level restoration in the rat

prostate. Recently, androgen-regulated formation and degradation of gap junctions have been demonstrated in androgen-responsive human prostate cancer cells (Mitra et al., 2006).

Concluding remarks

The studies on flutamide-treated pigs revealed that prenatal and neonatal changes in androgen action result in altered expression of AR gene and junction protein genes later in adult life, acting on selected cellular targets, mainly Sertoli and germ cells, thus leading to disturbed germ cell development and, in consequence, decreased male reproductive capacity of the pig. Of concern, neonatal window seems to be the most critical for the organization of BTB and consequently for normal spermatogenesis in the boar. Altered expression of junction proteins after flutamide treatment is likely related to insufficient testosterone production and/or excessive estradiol synthesis which may result from impaired Leydig cell function (Kotula-Balak et al., 2012). This suggestion sounds possible since the prevalence of reproductive organ pathologies increases with age when circulating testosterone levels decline

Table 1. Outline of our own studies on adult boars neonatally treated with flutamide.

Adult boar tissues	Effects of flutamide		
TESTIS	mRNA expression	Protein expression	References
ZO-1	↓	↓	Hejmej et al. 2012
N-cad	↓	↓	
ß-cat	↓	↓	
AR	↔	↔	
CYP19A1	↑	↑	Kotula-Balak et al. 2012
Cx43	↓	↓	Kopera et al. 2011
Germ cell sloughing	↑		
Apoptotic cell death frequency	↑		
EPIDIDYMIS	mRNA expression	Protein expression	References
ZO-1	↓	↓	unpublished
E-cad	↓	↓	Gorowska et al. 2014
ß-cat	↓	↓	
ST5AR2	↓	↓	
CYP19A1	↑	↑	
Cx43	↓	↓	Lydka et al. 2011
AR	↓	↓	
Apoptotic cell death frequency	↑		Lydka et al., 2012
Head sperm abnormalities	↑		
Sperm plasma membrane integrity*	↓		
Sperm plasma membrane stability*	↓		
Sperm mitochondrial oxidative capability*	↓		
PROSTATE	mRNA expression	Protein expression	References
E-cad	↓	↓	Gorowska et al. 2014
ß-cat	↔	↑	
ST5AR2	↔	↔	
CYP19A1	↓	↓	
Cx43	↑	↑	Hejmej et al. 2013
AR	↓	↓	
Apoptotic cell death frequency	↑		
SERUM HORMONE CONCENTRATIONS			References
Testosterone	↓		Kotula-Balak et al. 2012
Estradiol	↑		

↓, decrease; ↑, increase; ↔, no statistically significant change; Intercellular junction proteins: Zonula occludens (ZO-1), N-cadherin (N-cad), ß-catenin (ß-cat), Connexin 43 (Cx43), androgen receptor (AR), 5α-reductase (ST5AR2) and aromatase (CYP19A1). *: Data from *in vitro* experiments.

(Hsing et al., 2000; Feldman et al., 2002).

Most studies from other laboratories were performed on laboratory rodents, mainly rats. However, studying the effects of anti-androgen in species other than laboratory rodents has a rationale. The pig may better mimic the human system and have a closer similarity in physiology. It has both a longer gestation period and longer prepubertal period than laboratory rodents. Although significant progress in understanding a role of intercellular junction proteins on testicular function has been made there are still limited data on the involvement of androgens in cell to cell communication on epididymal and prostatic function. However, a decrease in AR mRNA or protein expression in each of the flutamide-treated tissues resulted in their morphofunctional alterations in adulthood (for detail see Table 1.). Thus, it is concluded that the dynamic cross-talk between the cells of adult testis, epididymis, and prostate is hormonally regulated *via* the AR signaling and involved in the control of spermatogenesis and maintenance of endocrine organ homeostasis. On the other hand, the possibility of direct action of flutamide *via* AR-independent pathway cannot be ruled out since the results of the study by Lee et al. (2002) on AR-negative prostate cancer cells suggest that flutamide may act through the activation of Ras-MAP kinase signaling pathway.

Acknowledgements. The authors would like to acknowledge current and previous members of the Department of Endocrinology, Institute of Zoology, Jagiellonian University for their work and involvement in the presented studies. Special thanks are expressed to Dr. Marta Zarzycka for illustrations. This work was supported by the HARMONIA3 project (No. 2012/06/M/NZ4/00146) from the National Science Centre.

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Accepted January 31, 2014