Blackwell Publishing

Asian J Androl 2007; 9 (4): 545–553
DOI: 10.1111/j.1745-7262.2007.00316.x

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·*Review*·

Androgenic regulation of novel genes in the epididymis

Bernard Robaire[1,3], Shayesta Seenundun[1], Mahsa Hamzeh[1], Sophie-Anne Lamour[1]

[1]Department of Pharmacology and Therapeutics and [2]Department of Obstetrics and Gynecology, McGill University, Montréal, QC H3G1Y6, Canada

Abstract

The epididymis is critically dependent on the presence of the testis. Although several hormones, such as retinoids and progestins, and factors secreted directly into the epididymal lumen, such as androgen binding protein and fibroblast growth factor, might play regulatory roles in epididymal function, testosterone (T) and its metabolites, dihydrotestosterone (DHT) and estradiol (E2), are accepted as the primary regulators of epididymal structure and functions, with the former playing the greater role. To ascertain the molecular action of androgens on the epididymis, three complementary approaches were pursued to monitor changes in gene expression in response to different hormonal milieux. The first was to establish changes in gene expression along the epididymis as androgenic support is withdrawn. The second was to determine the sequence of responses that occur in an androgen deprived tissue upon re-administration of the two metabolites of T, DHT and E2. The third was to study the effects of androgen withdrawal and re-administration on gene expression in immortalized murine caput epididymidal principal cells. Specific responses were observed under each of these conditions, with an expected major difference in the panoply of genes expressed upon hormone withdrawal and re-administration; however, some key common features were the common roles of genes in insulin like growth factor/epidermal growth factor and the relatively minor and specific effects of E2 as compared to DHT. Together, these results provide novel insights into the mechanisms of androgen regulation in epididymal principal cells. *(Asian J Androl 2007 July; 9: 545–553)*

Keywords: epididymis; cell culture; androgen withdrawal; dihydrotestosterone; estradiol; apoptosis; survivin; insulin like growth factor

1 Introduction

As early as 1926, pioneering studies by Benoit [1] demonstrated that the epididymis was dependent on an unknown testicular substance for maintenance of its structure and functions. This regulatory substance was identified as testosterone (T) [2]; there have been numerous publications on the response of the epididymis in mammals to androgen withdrawal (for review see [3]). Several lines of evidence have established that the active androgen in the epididymis is not T, but its 5α-reduced metabolite, dihydrotestosterone (DHT) [3, 4]; hence, steroid 5α-reductase activity is a key regulator of androgen action in this tissue [3–5]. In addition to androgens, many other hormonal factors have been postulated to play a role in regulating epididymal function [3, 6]. Of special note is estradiol (E2), another metabolite of T. Administration of estrogens has major effects on the epididymis [7–9]. With the advent of specific aromatase inhibitors and knockout mice for the two isoforms of the estrogen receptor, it has become possible to resolve some of the functions regulated by E2 in the epididymis.

2 Androgen and estrogen receptors in the epididymis

The most prevalent androgen, T, is produced by the interstitial cells (Leydig cells) of the testis on an ongoing basis. Concentrations of T in the seminiferous tubules are 10–100 times those found in serum [5, 10], and are essen-

Correspondence to: Dr Bernard Robaire, Department of Pharmacology and Therapeutics, 3655 Promenade Sir William Osler, Room 104, Montréal, QC H3G1Y6, Canada.
Tel: +1-514-398-3630 Fax: +1-514-398-7120
E-mail: bernard.robaire@mcgill.ca

tial for the maintenance of spermatogenesis [11–13]. High concentrations of T leave the testis through the efferent ducts and, upon entry into the epididymis, are rapidly converted to DHT [10], the most biologically active endogenous androgen, by the enzyme steroid 5α-reductase and to E2 by the P450 enzyme, steroid aromatase, which is found in spermatozoa [14].

The actions of both T and DHT are primarily mediated by binding to the androgen receptor (AR), although there is some evidence for cell-surface mediated androgen action [15, 16]. Androgen receptors are found in the epididymis of all species examined to date (rat, rabbit, dog, ram, monkey and human) (for review see [3]). Although only a single AR gene (approximately 110 Kb) has been identified, polymorphisms of this gene have been reported [17–20]. There are small changes in the concentrations of the mRNA for the AR along the epididymis [21]; when these occur, they are usually coincident with a small decline in the concentration of AR protein down the epididymis [22]. In the rat and other species, immunoreactivity to the AR is found throughout the duct, primarily in principal cells [23], although other cell types (basal and apical) also react [24, 25].

Clear evidence for the existence of estrogen receptors (ER) in the epididymis of many species has existed since the 1970s (for review see [3]). However, it is only with the advent of highly specific antibodies and knockout (KO) mice for the estrogen receptors, ERα and ERβ, that proof of the presence of the receptors and the need for E2 in the male excurrent duct system became evident [26]. The immunolocalization of ERα and ERβ along the efferent ducts and epididymis of the human and rat reveals that ERα is primarily expressed in the efferent ducts and the proximal end of the epididymis, whereas ERβ is found along the entire tissue [23, 24, 26]. The ERα KO mouse [27] is infertile owing to back pressure atrophy of the seminiferous tubules, which results from the inability of the efferent ducts and initial segment of the epididymis to re-absorb the large volume of fluid secreted by the testis. In contrast to the clear role of the ERα receptor, the function of ER in the epididymis is unclear [28].

3 Effects of androgen withdrawal on the epididymis

The main approach to understanding the effects of androgen withdrawal on the epididymis has been removal of the testes. Removing the testes, as the main source of androgen and other testicular factors that have direct input into the epididymis, serves to help resolve how the epididymis is normally sustained; it also provides insight into pathological conditions, such as hypogonadism [29], and a better understanding of the consequences of male aging (andropause), when androgen production shows a marked decline [30]. It is clear that this approach causes loss of not only androgens but also estrogens and any other testicular factors that might affect the epididymis; however, most of the effects of orchidectomy has been ascribed to the precipitous fall in androgens [31, 32]. Orchidectomy causes a decrease in epididymal weight that is less marked than that of sex accessory tissues, such as the prostate or the seminal vesicles [33]. Unlike other androgen-dependent male reproductive tissues, T replacement in orchidectomized animals, even at supraphysiological levels, only partially restores epididymal weight; presumably, this is because of the large proportion (nearly half) of epididymal weight that is attributable to spermatozoa and the luminal fluid bathing them [33, 34]. In the androgen-deprived state, spermatozoa become immotile, lose the ability to fertilize and die [35]. After orchidectomy, the epididymal luminal diameter and epithelial cell height decrease and there is a relative increase in the intertubular stroma [36]. The smooth endoplasmic reticulum content is dramatically reduced, whereas the extent of the decline in the Golgi apparatus is less pronounced [36–38]. Morphological changes in principal cells suggest that these cells are particularly sensitive to androgen levels, in contrast to the other epithelial cell types [39]. The secretory function of principal cells becomes compromised in the androgen-deprived state. Principal cells undergo a striking loss of apical microvilli from their surface, as well as lysosome accumulation, vacuolization, disappearance of vesicles from the cell apex, and increased endocytosis [39].

Withdrawal of androgen by orchidectomy induces a wave of apoptotic cell death in the epididymis, beginning in the initial segment and moving over several days to the cauda epididymidis [31, 40]. Apoptosis in the initial segment seems to be caused by withdrawal of androgen and of luminal components coming from the testis and is p53-independent [31, 40]. Using the entire epididymis, expression of Bcl-2, an anti-apoptotic factor [41], was suppressed by orchidectomy, followed by the appearance of Fas and DNA fragmentation, suggesting that regression of the tissue might be regulated via the Fas pathway [42].

To gain a better appreciation of the range of effects of androgen withdrawal on the epididymis, gene expression profiling has been used in several studies [41, 43]. The first of these studies, showing changes in the pattern of gene expression along the epididymis over the first week after orchidectomy, was done in the rat [41]. A transient upregulation in the expression of a select family of genes along the epididymis was found. Several an-

drogen-repressed genes (e.g. glutathione peroxidase-1 [Gpx-1]), showed increased expression in the epididymis after orchidectomy, whereas transcripts for many others (e.g. glutathione S-transferases and calcium-binding proteins) declined throughout the epididymis after orchidectomy. Other genes coding for metabolism-associated proteins, transporters and alpha-1 acid glycoprotein showed segment-specific regulation in the epididymis after orchidectomy. The expression of several previously uncharacterized heat shock proteins and apoptosis-associated genes was also found to change dramatically. Using the mouse, and observing changes at only one fixed time point, Chauvin *et al*. [43] confirm several of these findings.

To investigate the early response of apoptosis and survival genes that was activated after withdrawal and/or immediate supplementation of androgen in the different regions of the epididymis, we used rat apoptosis-specific arrays (ORN-012, SuperArray) that contained 96 genes. Male Brown Norway rats were orchidectomized and treated with empty or T-filled implants designed to maintain serum T at control concentrations. Rats were killed shortly (12 h and 1 day) after orchidectomy and epididymides were collected. The time-dependent and region-dependent changes in gene expression on this array are shown in Figure 1.

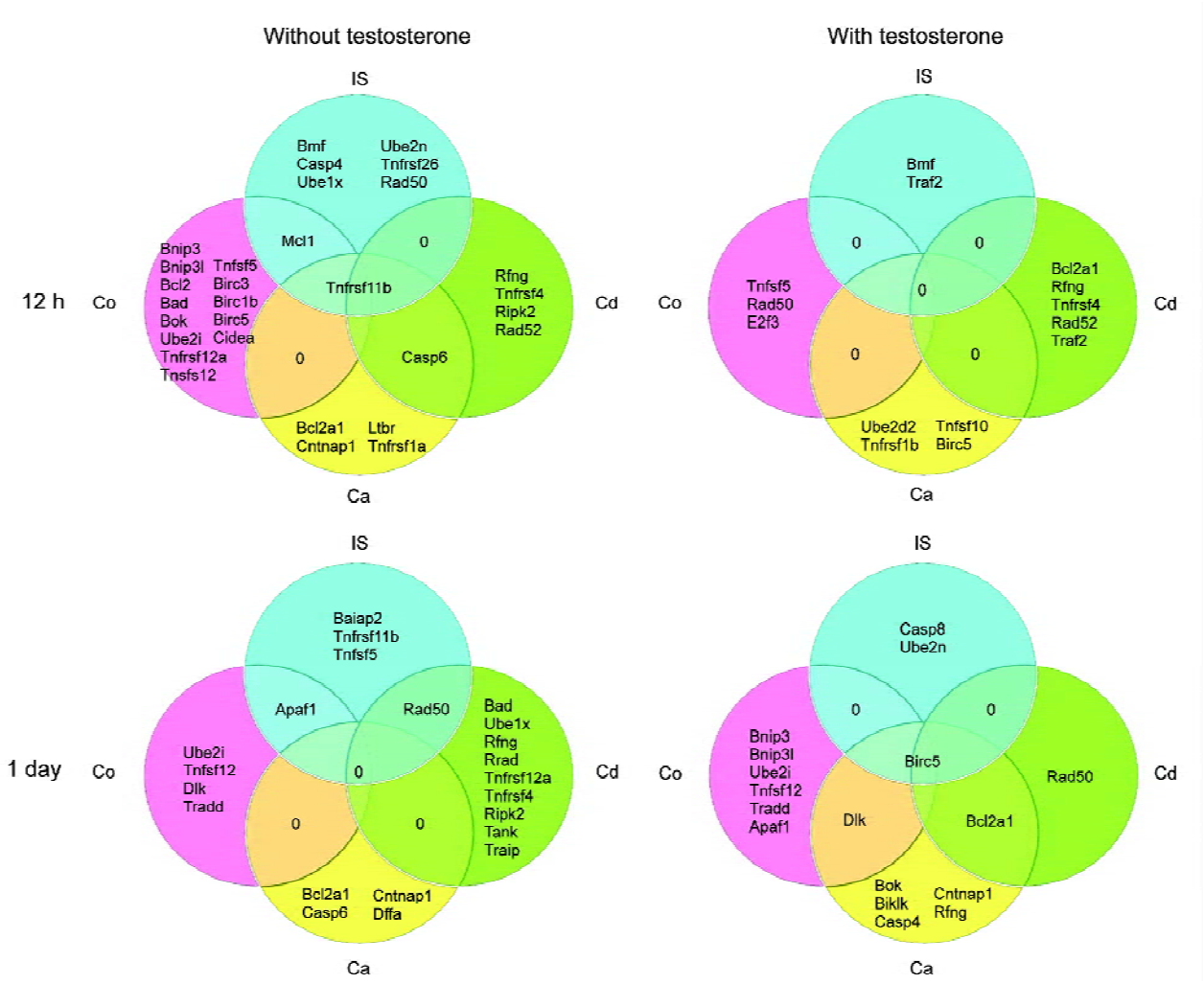

Figure 1. Region-specific alterations in gene expression at 12 h and day 1 in adult Brown-Norway rats after orchidectomy and immediate s.c. implantation of either empty or testosterone-filled polydimethylsiloxane implants designed to maintain serum testosterone (T) in the normal physiological range. Gene expression profiles were determined using the rat apoptosis-specific arrays (ORN-012, SuperArray). Five replicates using tissues from different animals were made for each region and each condition. Changes in gene expression were considered significant if they were increased or decreased by at least 1.5-fold in three of five replicates. IS, initial segment; Ca, caput epididymidis; Co, corpus epididymidis; Cd, cauda epididymidis.

In all treatment groups, less than 16% of the genes on the array showed a differential expression as compared to the control. In the absence of T, 6–16% of the genes were differentially expressed; the corpus and cauda epididymidis were the regions most affected, at 12 h and 1 day, respectively. T maintenance partially prevented differential expression of genes, with only 4–9% of the genes remaining differentially expressed.

At 12 h without T, most of the genes affected belonged to the tumor necrosis factor receptor (Tnfrsf) and Bcl-2 families. In the initial segment, Tnfrsf11b and Tnfrsf26 were downregulated, whereas all the other Tnfrsf members were upregulated in the caput, corpus and cauda epididymidis. No differentially expressed Bcl-2 family members were seen in the cauda epididymidis, whereas Bmf, Mcl-1 and Bcl2a1 were upregulated in the initial segment and caput epididymidis. In the corpus, Bcl-2, Bnip3, Bnip3l, Bad and Bok were all downregulated. T supplementation prevented the differential expression of all the Bcl-2 and Tnfrsf family members, except for Bmf, Tnfrsf4 and Tnfrsf1b, which remained upregulated.

At day 1 without T, the initial segment showed no genes differentially expressed, whereas Bad, Bnip3 and Bnip3l were downregulated in the cauda epididymidis. No additional Tnfrsf members were affected in the caput and corpus regions, whereas Tnfrsf11b/osteoprotegerin remained downregulated in the initial segment. In addition, the inhibitor of apoptosis protein, Birc5/surviving, was downregulated in all regions except the cauda, whereas all regions except the caput showed an increased expression of two repair proteins, Rad50 and Rad52. T supplementation did not affect the pattern of differentially expressed genes belonging to the Bcl-2 family. Furthermore, Tnfrsf11b remained downregulated in the initial segment, but became upregulated in the caput, whereas Birc5 became downregulated in the cauda.

These data, summarized in Figure 1, demonstrate that there are rapid region-specific responses that result in both selective upregulation and downregulation of selected members of the Tnf and Bcl families. The normally sustained repression of several genes associated with apoptosis might account for the normal lack of cell death seen in this tissue and the reported lack of spontaneous carcinomas originating in the epididymis [44].

4 Effects of hormone replacement on the regressed epididymis

Once the epididymis has regressed, administration of T has the ability to dramatically restore most of the histological features of the epithelium. Clearly, the lack of spermatozoa and fluid prevents a return to control organ weight [33]. Unlike the case with the prostate, giving very high doses of T does not result in epithelial hyperplasia [33]. Relatively few studies have examined the sequence of events that occurs upon re-administration of androgens once the epididymis has regressed. We have found that after administration of DHT to rats that had been orchidectomized for 1 week, no significant increase in weight occurs at day 0.5 or 1, the first significant increase is seen at 3 days, and by 7 days the weight has nearly doubled; interestingly, E2 administration had no effect. Using orchidectomy and hormone replacement after a one-week regression period, we examined the actions of the two active metabolites of T, DHT and E2, on the sequence of gene expression responses in the epididymis to resolve the molecular events associated with androgen action in this tissue.

Using Affymetrix Rat Genome 230-2 Microarray chips containing 31 000 probe sets, gene expression profile changes were observed in the initial segment and caput of the regressed rat epididymides as early as 0.5, 1 and 7 days after replacement with either DHT or E2. Differential expression was defined as those transcripts that had a difference of twofold or greater in normalized values and were statistically different (significance level set at $P < 0.05$); microarray results for a selected subset of genes were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR).

Interestingly, of the 1 059 genes that were affected by orchidectomy after 7 days, the expression of approximately as many genes (546 genes) were upregulated as were downregulated (513 genes). After treatment for 7 days with DHT, over 75% of the genes returned toward the control, un-orchidectomized values. In contrast, the expression of only 13 genes was altered by E2 treatment, and only 5 of these genes had been affected by orchidectomy. Among genes affected by DHT replacement after androgen deprivation, many genes belong to specific functional gene families. These include solute carrier family (*Slc12a3, Slc15a2, Slc22a5, Slc9a2*), cell communication (*Gja1, Gja4, Gjb3*), cell growth, regulation of cell proliferation and apoptosis (*Ahr, Ar, Plau, Pdgfc, Figf*), signal pathway and signal transduction (*Ramp3, Calcrl, Itpr3*), proteolysis and peptidolysis (*Adam7, Adam9, Ctsc*), and development (*Acta1, Gas7, Pppr2b2*). We shall focus on three genes affected by DHT and one by E2.

Placentae and embryos oncofetal gene (*Pem*), a member of the homeobox factor family, is expressed in Sertoli cell of the testis and in the epididymis, suggesting that it plays an important role in sperm function [45]. Using transgenic mice that express *Pem* in Sertoli cell during all stages of the seminiferous cycle, *Pem* was

shown to regulate the expression of Sertoli cell genes that encode proteins that serve to control premeiotic DNA replication, DNA repair and chromatin remodeling in the adjacent germ cells [46]. Although androgens are necessary but not sufficient for *Pem* expression in Sertoli cells, we found that DHT was able to maintain the level of *Pem* expression in the epididymis; the significant decrease found after orchidectomy was reversed and returned to the control level after replacement with DHT for 1 day.

Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform (Ppp2r2b), regulates the activity of the PP2A catalytic subunit in spermatids, and is involved in spermatogenesis, especially spermatid elongation [47]. Although the mRNA for this gene has been shown to be expressed in the rat brain and testis, it has not been looked at in the epididymis. We found that this gene was highly upregulated after orchidectomy, and that DHT suppressed its expression to control levels after 7 days.

Cysteine-rich secretory protein 1 (Crisp1), an androgen-regulated secretory protein of the rat epididymis, binds to the post-acrosomal region of the sperm head [48]; therefore, it is one of the sperm surface proteins thought to be involved in the fusion of sperm and egg plasma membranes. We found that DHT treatment for 7 days reversed the suppression of expression caused by orchidectomy to the control levels.

Although the expression of many genes is affected by DHT, few genes responded to E2. Chloride intracellular channel 3 (LOC 296566) (Clic3) expression significantly declined after orchidectomy; this change could be reversed by E2 after 7 days. CLIC proteins have been identified in bovine epididymal spermatozoa [49] and are likely to play significant roles in sperm function.

Therefore, it is clear that an androgen-dependent regressed tissue, such as the epididymis, mounts a robust, rapid response after exposure to DHT by both upregulating and downregulating a number of genes well before any structural changes are apparent; the response to DHT is far more dramatic than that of E2.

6 Changes in gene expression in immortalized mouse caput epididymal principal cells in response to androgen withdrawal and re-administration

Several pure epididymal cell lines have been established recently [50–53]. The cell lines developed in the Orgebin-Crist lab (PC-1, DC-1, DC-2 and DC-3) are the first immortalized epithelial cell lines of the epididymis that have been shown to display tissue and initial segment/caput-specific gene expression [51]. They show a similar polarity to principal cells *in vivo* in terms of their distribution of cellular organelles, and retain the type of junctional complexes seen between principal cells *in situ*. They also express a number of principal cell markers and are responsive to androgens.

To gain a comprehensive insight into gene expression in following androgen withdrawal and supplementation, we explored the androgen dependence of the PC-1 epididymal cell line by using DNA microarrays [54]. Changes in gene expression occurring 2, 4 and 6 days after androgen deprivation and 2 days after androgen supplementation after being deprived of androgen for 2 or 4 days were examined. Four distinct patterns of gene expression were activated following androgen withdrawal; the vast majority of genes displayed an early or late transient increase in expression levels. A differential ability of rescue was seen among androgen-regulated genes depending on the time of androgen supplementation. Many of the genes that were rescued at 4 days were functionally linked by direct interactions and converged on insulin like growth factor-1 (IGF-1). The ability for rescue after 4 days of androgen deprivation was severely compromised in many genes belonging to specific functional gene families (cell adhesion, cell growth, apoptosis and cell cycle) and might be mediated in part by changes in AR coregulator expression. Using a pathway analysis approach, a common node of several of the affected genes was IGF-1 (Figure 2).

7 Potential role of the insulin like growth factor gene (IGF) family and epidermal growth factor (EGF) in mediating androgen action

The insulin-like growth factor network is composed of IGF-1 and IGF-2, mitogenic peptides involved in the regulation of cellular proliferation, differentiation and apoptosis [55]. IGF have a structural homology with proinsulin, and are mainly produced by liver and bone marrow [56]. Both IGF-1 and IGF-2 bind with high affinity to the IGF-1 receptor, a tyrosine kinase located on the cell membrane, and initiate mitogenic responses in cells [57, 58]; IGF exert anti-apoptotic and mitogenic effects in the prostate [59]. Similarly, IGF-1 plays a crucial role in Leydig cell maturation in the testis; null mutations for this growth factor result in decreased levels of androgens, a poorly developed epididymis, and infertility [60, 61].

A physiological role for IGF-1 in the regulation of epididymal functions was hypothesized over 10 years ago as judged by the varied immunohistochemical localization of IGF-1 protein in the rat epididymis [62]. IGF-1R mRNA and protein have also been localized in the epididymis [60, 63]. Adult mice with a homozygous null mutation of the

IGF-1 gene are infertile dwarfs with reduced T production and, consequently, reduced spermatogenesis and reproductive organ size [60]. Interestingly, the epididymal phenotype of IGF-1 null mice is more severe in the distal regions of the tissue, with greater reductions in weight and decreased tubule coiling. We found that the expression of IGF-1 and IGF-1R was highest in the distal epididymal regions [64]. These results are consistent with a role for IGF signaling in the distal regions of the epididymis (corpus and cauda epididymidis). Further corroborating this conclusion is the demonstration of the unique effects of 5α-reductase inhibitor treatment on the expression of IGF-1, and possibly IGF-1R, predominantly in the corpus and cauda epididymidis [64].

IGF-binding proteins (IGFBP1-6) modulate the availability of unbound IGF for interaction with IGF-1R [65]. IGFBP-3 is the most prevalent of the six IGFBPs [66]. More than 90% of circulating IGF is bound to IGFBP-3. IGFBP-3 is known to impair IGF action and inhibit cell growth, either by blocking free IGF or through an IGF-independent mechanism [67]. IGF and IGFBP-3 play important roles in prostate epithelial cell proliferation, apoptosis and tumor progression [68]. *In silico* analysis identified that a putative androgen response element in the IGFBP-3 promoter and 10 nmol/L of DHT (a growth inhibitory dose) can induce IGFBP-3 expression in LNCaP cells [69]. We analyzed the epididymal expression of two high-affinity IGF binding proteins, IGFBP-5 and IGFBP-6. Again, these genes were more highly expressed in the distal regions of the epididymis and were mainly affected by 5α-reductase inhibitor treatment in these regions. IGFBP-5 expression was particularly affected by treatment, decreasing dramatically in the cauda epididymidis [64].

IGF-1 and EGF play synergistic critical roles in pathological processes, such as carcinogenesis and wound healing [70]. The presence of EGF [71] and EGF receptors [72] in the testes of different species implicates their central role in the regulation of spermatogenesis [71, 73]. It was shown that removal of the submaxillary gland, the tissue that produces most EGF, results in a major loss of

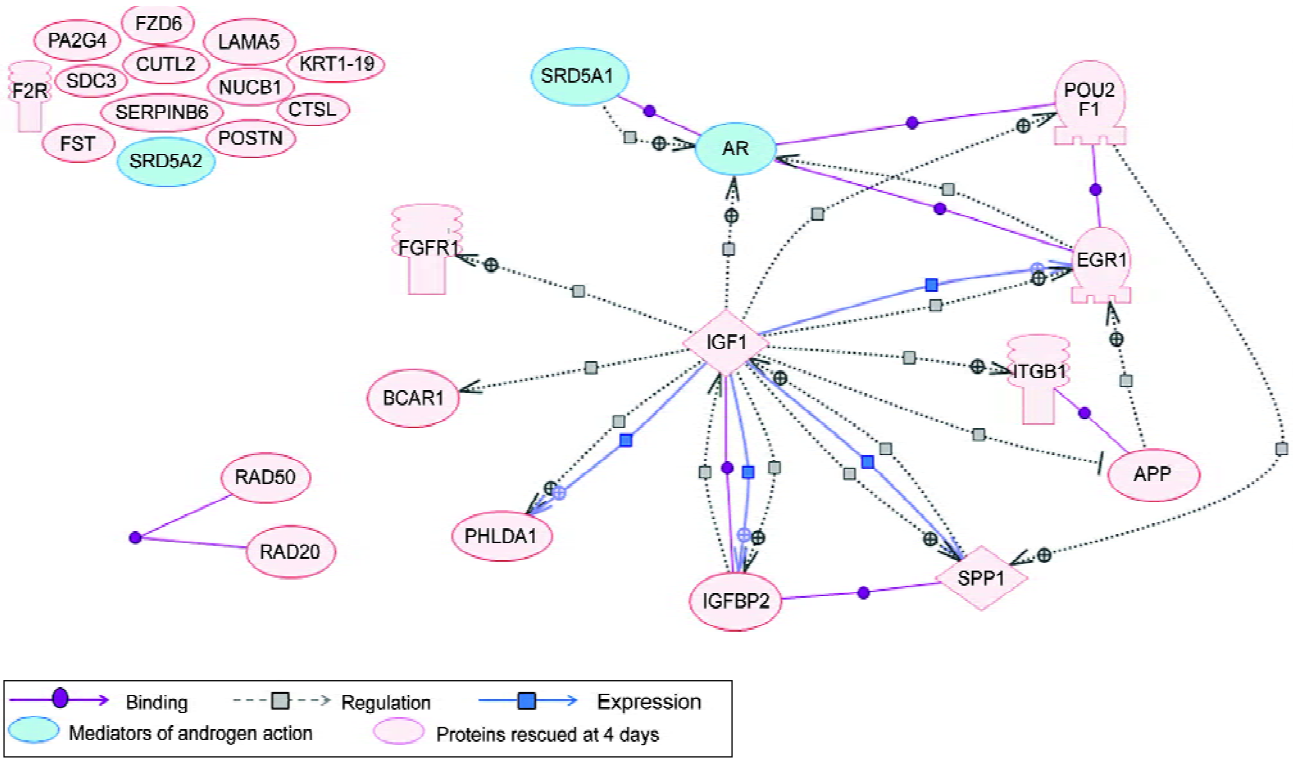

Figure 2. Potential direct functional linkages between genes rescued by dihydrotestosterone (DHT) supplementation after 2 days of androgen deprivation. Genes significantly changing by at least 1.5-fold after androgen withdrawal and rescued by DHT supplementation at 4 days were used as the starting group. Mediators of androgen action were also included in the analysis. Only genes that have been directly linked to one another through a primary interaction in the literature to date are shown. Arrows indicate the direction of the interactions, + indicates positive influence, T junctions indicate inhibition, and the color and shape of the linkages indicate the type of interaction between the two gene products (expression, regulation, binding or phosphorylation). Modified with permission from Seenundun and Robaire [54].

spermatozoa from the epididymis that was reversed by giving EGF; no effects on serum T or gonadotropins were noted, implying a direct role of this growth factor in regulating epididymal function [74]. Androgens increase EGF binding sites in the rat prostate and modulate the cellular proliferation of this tissue [75]; therefore, suppression of EGF receptor signaling results in reduction in the incidence of prostate cancer metastasis [76]. The EGF receptor in the epididymis of non-human primates is located in both the basolateral and apical borders of the epididymal epithelial cells [77]. Furthermore, immunohistochemical studies on C3H mice have shown that the intracellular localization of the EGF receptor varied along the epididymis; the staining was more intense in the cytoplasm of principal cells in the caput and in the apical cytoplasm in the corpus and cauda regions [78]. The *EGF* gene is expressed in the mouse epididymis in a segment-specific manner: low in the proximal region of the caput and increasing in the corpus [79]. However, immunolocalization of EGF, IGF-1, IGF-1R and IGFBP-3 in the adult rat epididymis has not been clearly established.

The underlying mechanisms regulating IGFBP-3 expression and its functional role in epididymal cell growth remain to be elucidated. We speculate that EGF/IGF-1 interact intimately with androgens to regulate cell growth and proliferation. By taking a series of complementary approaches to understand how the action of T and its metabolites are mediated, both when initially withdrawn and re-administered to the epididymis, we have identified several novel gene targets. Some of these genes are likely to be early steroid hormone response genes and might prove to be powerful targets for the development of drugs that will regulate epididymal functions for purposes of controlling male fertility.

Acknowledgment

These studies were supported by a grant from the Canadian Institutes for Health Research.

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