Abstract

The placenta and tumors share important characteristics, including a requirement to establish effective angiogenesis. In the case of the placenta, optimal angiogenesis is required to sustain the blood flow required to maintain a successful pregnancy, whereas in tumors establishing new blood supplies is considered a key step in supporting metastases. Therefore the development of novel angiogenesis inhibitors has been an area of active research in oncology. A subset of the molecular processes regulating angiogenesis are well understood in the context of both early placentation and tumorigenesis. In this review we focus on the well-established role of androgen regulation of angiogenesis in cancer and relate these mechanisms to placental angiogenesis. The physiological actions of androgens are mediated by the androgen receptor (AR), a ligand dependent transcription factor. Androgens and the AR are essential for normal male embryonic development, puberty and lifelong health. Defects in androgen signalling are associated with a diverse range of clinical disorders in men and women including disorders of sex development (DSD), polycystic ovary syndrome in women and many cancers. We summarize the diverse molecular mechanisms of androgen regulation of angiogenesis and infer the potential significance of these pathways to normal and pathogenic placental function. Finally, we offer potential research applications of androgen-targeting molecules developed to treat cancer as investigative tools to help further delineate the role of androgen signalling in placental function and maternal and offspring health in animal models.

Androgen dependent mechanisms of pro-angiogenic networks in placental and tumor development

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***Highlights**

- The placenta and tumors share important characteristics, including a requirement to establish effective angiogenesis.
- We focus on the well-established role of androgen regulation of angiogenesis in cancer and infer potential relevance to placental development and function.

Introduction

It has long been recognized that the placenta and tumors share important characteristics. These include mechanisms related to immune privilege and most notably in the context of this review, a requirement to establish effective neovascularization and angiogenesis. Placental angiogenesis is a tightly regulated process involving complex interactions of pro- and anti-angiogenic factors, which if dysregulated can lead to different pregnancy complications including preeclampsia [1]. Examples of important pro-angiogenic factors in the placenta include vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and fibroblast growth factor (FGF) [2], whereas soluble fms-like tyrosine kinase 1 (sFlt-1) is noted as a key anti-angiogenic factor [3]. A better understanding of placental angiogenesis would be beneficial in understanding pathological conditions such as preeclampsia and intrauterine growth restriction. This review will provide a summary of current understanding of the role of angiogenesis in cancer and placental physiology, with an emphasis on androgen regulation of pro-angiogenesis pathways.

Androgens have long been known to play essential roles in male embryonic development and pubertal maturation [4] and are now recognized as having a role in angiogenesis [reviewed in 5]. The most abundant physiological androgens in men are testosterone and its more potent derivative 5α-dihydrotestosterone (DHT) which is produced by steroid-5α-reductase enzymes [6]. Testosterone can also be converted to the primary estrogen (β-estradiol) by aromatase [7], therefore it is often essential to consider the relative roles of androgen and estrogen signalling. Androgen production is regulated in the hypothalamus, where gonadotrophin hormone-releasing hormone (GnRH) triggers the release of luteinizing hormone (LH) from the pituitary gland [8]. LH in turn acts on the testes where the majority of the testosterone is synthesized. Testosterone is transported to target tissues primarily bound to the sex hormone-binding globulin or to albumin [9, 10]. Secondary androgens, such as androstenedione (AED) and dehydroepiandrostenedione

(DHEA) are produced primarily by the adrenal glands [8]. As we will discuss in detail later, there is also evidence of androgen synthesis [11] and androgen receptor (AR) expression in the placenta and endometrium [12-14].

Androgen receptor signalling

The actions of androgens are mediated primarily by the AR, also referred to as NR3C4 [15]. The AR is a member of the ligand dependent superfamily of nuclear receptor transcription factors which, in the presence of androgens, regulates the transcription of target genes [15]. Nuclear receptors consist of three major domains: the N-terminal region, the DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) [16]. The N-terminal region is variable in both sequence and size and in the AR harbors an agonist independent transcriptional activation function (AF-1) [17]. The highly conserved DBD is situated in the centre of the polypeptide and selectively and preferentially binds to androgen response elements in the regulatory regions of androgen target genes. The DBD and LBD are separated through a variable hinge region that contains DNA minor-groove binding residues [18]. The LBD is the site where both ligands and coregulators bind and where the second transcriptional activation function (AF-2) region is situated. In contrast to AF-1, AF-2 is ligand-dependent and full transcriptional activity can only be accomplished when AF-1 and AF-2 act together [19]. The AR regulates gene expression by recruiting multiple epigenetic coregulators, often through a conserved LxxLL motif, which control transcription via covalent histone modifications (Figure 1) [20]. The role of coregulators in gene activation and how these relate to the modulation of histone lysine acetylation and methylation is an area of active research. Nuclear receptor-coregulator complexes, and by inference the AR-coregulator complex, are believed to be dynamic [21] and involve the recruitment of diverse enzymes which covalently modify the N-terminal tail of histones such as lysine acetyltransferases (KATs), deacetylases (HDACs), lysine methyltransferases (KMTs) and lysine

demethylases (KDMs), kinases/phosphatases, poly(ADP)ribosylases and ubiquitin ligases [22]. KATs and HDACs have been intensively studied and the general paradigm is that KAT activity increases DNA accessibility, thus activating gene transcription, whereas HDACs are associated with transcriptional repression [23, 24]. It is important to note that certain coregulators, including KDM1A which is also expressed in the placenta [25], can exhibit transcriptional activation and repression properties in a cellular and epigenomic context-dependent manner [26].

Androgens and fetal development

During normal embryonic development and sex determination, the 46XY fetus instructs the primitive bipotential gonad to develop into testes [4]. Testicular androgen production and the ability to respond to these androgenic hormones are both then required to enable the XY fetus to complete male sex differentiation [4 and references therein]. Yet, it is estimated that between 1 in 20,400 and 1 in 99,100 infants are unable to respond to androgens and present with complete 46 XY sex reversal, termed complete androgen insensitivity [4]. Complete androgen insensitivity syndrome (CAIS) results in 46XY sex reversal and typically presents with pubertal amenorrhea or inguinal swelling in infants [27]. About 90-95% of all CAIS cases show mutations in the AR causing hormone resistance [28]. Partial androgen insensitivity syndrome (PAIS) is more common and the PAIS phenotype is much more complex and diverse [4]. We [29-32] and others [33] have identified and functionally characterized numerous loss of function and intronic mutations in the *AR* locus in individuals with complete and partial AIS. As we will explore in more detail later, the inability of the CAIS fetus and the fetal placenta component to respond to androgens suggests that pregnancy is sufficiently sustained by the ability of the maternal placental component to respond to androgens.

Androgens, angiogenesis and cancer

Much of our understanding of androgen regulation of angiogenesis has been obtained in cancer studies. Androgens and androgen signalling are implicated in many human cancer types, including prostate [34, 35], testicular germ cell [36] and bladder [37] cancers. Androgens are also known to have complex roles in breast tumors [38-40]. AR coregulators, including the lysine demethylase KDM1A/LSD1 [37, 41, 42] and p160 coactivators [43-45] have also been implicated in cancer, most notably prostate cancer (PCa). PCa is the most common non-cutaneous cancer affecting men [46]. The treatment options for PCa are often dependent upon the age and general health of the patient, as well as the stage and grade of the cancer. Watchful waiting, active surveillance, radical prostatectomy and radiotherapy remain the most effective initial therapies of localized PCa, however these can be associated with negative impacts on quality of life [47, 48] and post-treatment recurrence remains common [49]. In the case of PCa, treatments which block androgen biosynthesis or signalling, so called androgen deprivation therapies (ADT) are important treatments for advanced PCa (Figure 2). Existing ADTs target AR function by blocking androgen biosynthesis (GnRH analogues), acting as AR selective antagonists (bicalutamide, enzalutamide) or blocking intra-tumoral androgen biosynthesis (abiraterone) [50, 51]. Unfortunately, ADTs are ineffective in the long term for many patients, as incurable hormone refractory PCa tumors which are resistant to ADTs, commonly emerge within ~18 months at which point only palliative treatments are available. For this reason, great effort was invested to develop novel therapies targeting tumor angiogenesis. Indeed >20 years ago, Marshall and Narayan suggested a role for androgens in PCa angiogenesis [52]. Subsequent studies in mouse PCa xenograft models indicated castration decreased angiogenesis with a concomitant decrease in levels of vascular endothelial growth factor A (VEGFA)[53]. More recently, we and others found that androgens and AR-coregulators

regulate VEGFA levels (Figure 3) [35, 54, 55]. Consistent with this there is clinical [54] and genetic [56] evidence suggesting a link between VEGFA expression and poorer outcomes in PCa patients. Androgen depletion has been found to significantly induce apoptosis of tumor associated endothelial cells, suggesting a direct effect on angiogenesis, independent of the effect of androgen withdrawal on PCa cell proliferation and/or viability [53]. For these reasons there was much hope for treatments targeting pro-angiogenesis mediators such as VEGFA. However, clinical trials of angiogenesis inhibitors have been disappointing with only modest anti-tumor activity achieved in patients [57], though the use of anti-VEGFA therapy in combination with other agents shows more promise [58, 59].

Androgens and angiogenesis in endometrial and placental function

There is robust AR expression in the endometrium [13, 60] and both the AR and dihydrotestosterone are implicated in endometrial cancer. There is also evidence of endometrial and placental androgen biosynthesis [11, 12]. However the expression of AR in the placenta is controversial [14, 60-62]. In normal pregnancy, circulating androgen levels generally increase, compared with non-pregnant female hormone levels. Testosterone has been shown to increase by day 15 after the luteinizing hormone surge with reports of ~1.55 – 1.7 fold average increase from day 15 through to week 33 in comparison to non-pregnant women, changes were not observed prior to day 13 [63, 64]. Androstenedioine levels rise from day 14 and increase on average by 1.3 fold from week 5 to 40 in comparison to non-pregnant women [63, 64]. Additionally, testosterone decreased uterine blood flow to the placenta [65]. It is interesting to note that the free androgen index fell rapidly from weeks 5-21, plateauing at week 21 and rising marginally at 40 weeks [63]. Interestingly, aberrant placental function has not been described in the pregnancies of CAIS fetuses, suggesting that maternal androgen signalling may be sufficient to mediate any required androgen-regulated angiogenesis during placental

development. Excess testosterone during pregnancy can negatively impact placental angiogenesis [66, 67]. For example, androgen levels are higher in pregnant women with polycystic ovary syndrome (PCOS) as compared with normal pregnancy [68]. Free androgen index, testosterone, androstenedione, and dehydroepiandrosterone (DHEA) levels were all increased in PCOS pregnancies compared with normal pregnancies during weeks 22 to 28, but not earlier in pregnancy (weeks 10 – 16) [68]. Despite differing circulating levels of androgens during pregnancy, fetal virilisation was not observed. However this was likely due to fetal virilisation occurring between weeks 8 and 13 of gestation, whilst the increased levels of androgens were observed at week 16 [63, 64, 68]. The placenta also expresses aromatase which rapidly converts androgens to estrogen [68, 69]. This could explain why the fetus is not affected by virilisation in normal pregnancy. No associations have been observed between concentrations of testosterone and the sex of the baby in pregnant vs non-pregnant women [63]. Levels of DHEA, androstenedione or testosterone in normal pregnant women vs pregnant PCOS women were also not dependent on the sex of the baby [68].

Increased first trimester total testosterone levels in women was also shown to be an independent predictor of gestational diabetes mellitus (GDM) [70]. Increased androgen sensitivity in the human GDM placenta compared to healthy placentas has also been reported [69] as have increased AR mRNA and protein levels of in GDM placentae. In contrast aromatase protein expression was decreased in GDM placentas compared with healthy placentas, which was suggested to lead to reduced conversion of testosterone to estrogen [69]. Placentas from women with GDM also showed decreased human placental mRNA and protein expression of *VEGFR2* and *VEGFA* compared to control placentas. Qualitative analysis of immunohistochemical localization reported that although mRNA and protein levels were lower,

and immune-staining was weaker, VEGFR2 and VEGFA were expressed in the same cells and localities within the GDM and control placentas [67].

There is evidence that suggests the mechanisms of angiogenesis are similar in the placenta and prostate cancer. Evidence from early studies on first generation angiogenesis inhibitors such as TNP-470, implicated impaired angiogenesis as a contributing factor in intrauterine growth restriction of the fetus [71]. TNP-470 was shown to have an effect on human PCa cells and a number of tumors in patients [72, 73]. Similarly, the endogenous angiogenesis inhibitor, angiostatin4.5, has also shown activity in tumors [74]. Like TNP-470, angiostatin4.5 also reduces murine placental angiogenesis and with the offspring showing skeletal growth delays [75]. Maliqueo and colleagues have recently provided a comprehensive review of the diverse roles of the sex steroids in the regulation of the uterine-placental vasculature [76]. Yet current understanding of the role of androgen signalling in placental development and particularly its potential role in regulating angiogenesis in the placenta, is incomplete. Androgens are known to stimulate proliferation of human umbilical vein endothelial cells (HUVECs) [77], indicating a role for androgens during pregnancy. Interestingly, this androgen effect on HUVEC function was not sex dependent. There is also evidence from rat models that excess androgen reduces uterine blood flow and increases maternal and adult offspring blood pressure, by a convergence of mechanisms involving angiotensin II, reduced eNOS activity, a consequent reduction in NO production and AR activation of protein kinase C (PKCδ) [78-81]. Furthermore, increased testosterone results in elevated expression of hypoxia related genes including hypoxia inducible factor 1 α (HIF1α) [80], an established positive regulator of VEGFA [82]. VEGFA is believed to play important roles in the earliest stages of embryonic implantation [83]. Yet the potential role of androgens in regulating VEGFAand angiogenesis in the placenta remains poorly defined. But in a recent ovine study examining the effects of testosterone on the placenta, *VEGFA*

expression was observed to be androgen responsive. Indeed AR and the KDM1A coregulator are recruited to an androgen response element (ARE) in the ovine *VEGFA* locus [25]. On gestational day 90, placental *VEGFA* mRNA, placental VEGFA and AR protein levels increased in testosterone-treated ewes compared with control placentas [25].

Beyond androgen regulation of VEGFAin angiogenesis [35, 54], there is also evidence for a role for androgens in regulation of the Slt/Robo pathway [84]. The slits(1-3) are secreted glycoproteins act as ligands for the Robos(1-4) transmembrane receptors. In one recent study, expression of Slit and Robo mRNA was compared in normal and preeclamptic (characterised by impaired angiogenesis and hypoxia) human placental tissue specimens [1]. *Robo1* and *Robo4* were shown to have significantly higher expression in pre-eclamptic as compared to healthy tissue [85]. Additionally, hypoxia was shown to increase expression of Slit 2 in BeWo choriocarcinoma cells and Robo1 and 4 and Slit 3 in human umbilical vein endothelial cells (HUVEC) cells. Robo4 is a vascular specific and its activation by Slit2 has been shown in vitro to inhibit mouse lung endothelial cell migration, tube formation and permeability induced by vascular endothelial growth factor (VEGF)-165 [85]. Conversely, human malignant melanoma cells found to be expressing Slit2 were shown to induce angiogenesis in a xenograft animal model [86]. This effect was reversed, and tumour growth impeded, by Robo1 blocking antibodies or soluble Robo1 receptor. Slit/Robo signalling is implicated in multiple, often contradictory, ways in several cancers relating to invasion, migration and apoptosis as well as angiogenesis (Gara et al., 2015). In most cases the Slits and Robos are under expressed due to promoter hypermethylation. Indeed there is evidence that androgen excess during pregnancy can reduce Robo1 expression [84]. One consequence of this would be to impact angiogenesis.

Human trophoblast cells isolated at late stage pregnancy have been shown to express the angiogenesis inhibitor, pigment epithelium-derived factor (PEDF), at higher levels than those

from early pregnancy [87]. Additionally, only late stage pregnancy derived cells were capable of reducing angiogenesis of human placental endothelial cells. This anti-angiogenic effect could be reduced with the addition of a PEDF blocking antibody. Recombinant PEDF was also shown to induce an anti-angiogenic effect through inhibiting VEGFAsignalling. This suggests PDGF acts in a paracrine manner to slow angiogenesis in the latter stages of pregnancy. Expression of PEDF has also been shown to be reduced in PCa as compared to healthy control [88]. However, there is evidence that androgen can both activate [89] and reduce [88] PEDF expression in testicular peritubular cells and PCa respectively. Whether androgens regulate PEDF in the placenta remains unknown.

It is also worth noting that whilst the placenta is undergoing angiogenesis and remodelling, so is the maternal endometrium. The imbalance of pro- and anti-angiogenic factors has also been shown to play a major role in disorders such as preeclampsia, where vascular disruption is evident in both the placenta and maternal endothelium during this essential vascular remodelling period [90, 91]. A number of studies have indicated that a key component of circulating angiogenesis inhibitors is whether or not the vascular endothelial cells are quiescent or activated and therefore expressing Fas at higher levels [92].

Conclusion

In this review we have discussed the current understanding of androgen signalling and how this relates to angiogenesis in placental and cancer contexts. Previous studies have reported changes in androgen levels during pregnancy and in pathogenic processes including PCOS and GDM which are associated with concomitant changes in placental angiogenesis. However, further work is required to elucidate the complex role of androgens and their metabolites in

placental angiogenesis and development. The extensive repertoire of pharmacological inhibitors of androgen signalling developed for PCa represent excellent tools to interrogate the androgen signalling pathway in placental development. The availability of potent pharmacological agents which can inhibit androgen synthesis (abiraterone) and conversion to estrogen (aromatase inhibitors), coupled with AR-antagonists such as bicalutamide and enzalutamide (Figure 2), afford the potential to further delineate the complex roles of androgens in placental angiogenesis in animal models. Such approaches will also help advance understanding of the life-long consequences of deregulated androgen signalling *in utero*.

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Figure 1. (A) Crystal structure (PDB: 2AO6) of the AR ligand binding domain in complex with agonist R1881 and the LXXLL motif derived from SRC2/TIF2/NCOA2 [93]. The LBD is represented in cartoon format (green) and shows the three layer antiparallel alpha-helical sandwich conformation typical of NRs. The SRC2/TIF2/NCOA2 coactivator peptide is shown in yellow and adopts an alpha helical conformation. Conserved leucine residues are shown in cyan and contact the cofactor binding cleft on the LBD surface. The ligand R1881 is shown in red with the ligand binding pocket. (B) Crystal structure (PDB: 1R4I) of the rat AR DNA binding domain (DBD) bound to the direct repeat of the hexamer AGAACA as a direct repeat, separated by thee nucleotides (DR3). [94]. The double stranded DNA duplex is shown in wireframe. The DBD dimer is represented in cartoon format (green) and zinc atoms are portrayed as grey spheres. The DBD monomers adopt alpha-helical conformations of which one these, the DNA recognition helix, contacts specific bases and sugar-phosphate backbone of the 'response element'. Interactions between the DBD monomers stabilise the dimer.

Figure 2. Androgen deprivation therapies are important treatment approaches for advanced prostate cancer. Abiraterone blocks adrenal and gonadal androgen biosynthesis by inhibiting the Cyp17/17-a-hydroxylase/C17,20 lyase enzyme. Flutamide, bicalutamide and enzalutamide block androgen signalling by acting as AR antagonists. ARN-509, also termed JNJ-56021927 is in clinical phase III trials for advanced PCa (clinicaltrials.gov accessions: NCT02772588, NCT02489318, NCT02123758, NCT02578797, NCT01946204, NCT01790126, NCT01792687, NCT02106507, accessed November 10, 2016).

Figure 3. Evaluation of the expression of vascular endothelial growth factor (VEGF-A) in prostate cancer specimens as previously reported (Wegiel et al., 2008). Representative staining examples are provided for benign prostate hyperplasia (BPH), low and high grade malignant prostate tissue**.** Reproduced with permission from Kashyap et al [54] in Molecular Oncology, 2013 Jun;7(3):555-66. doi: 10.1016/j.molonc.2013.01.003; Elsevier.

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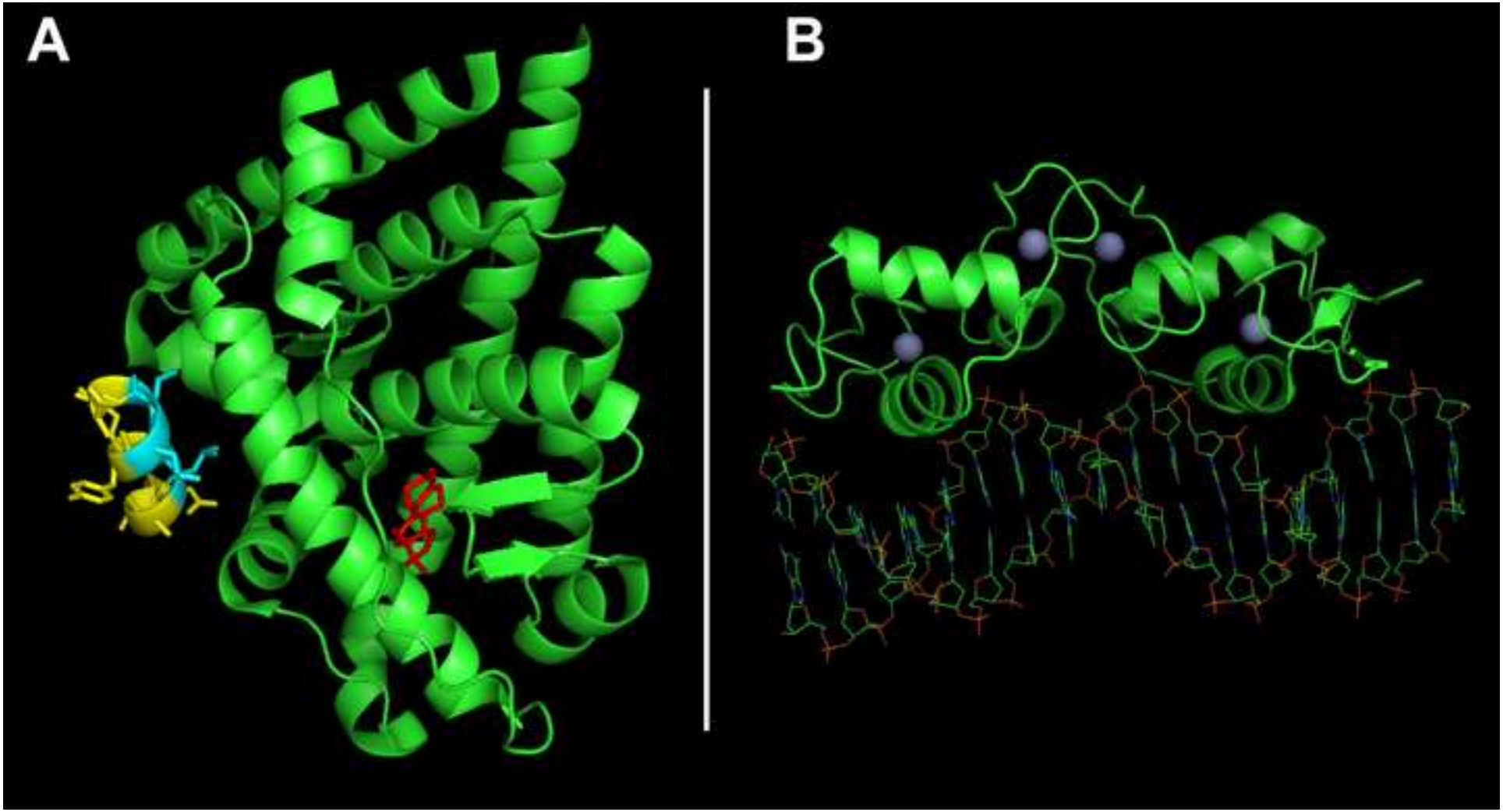

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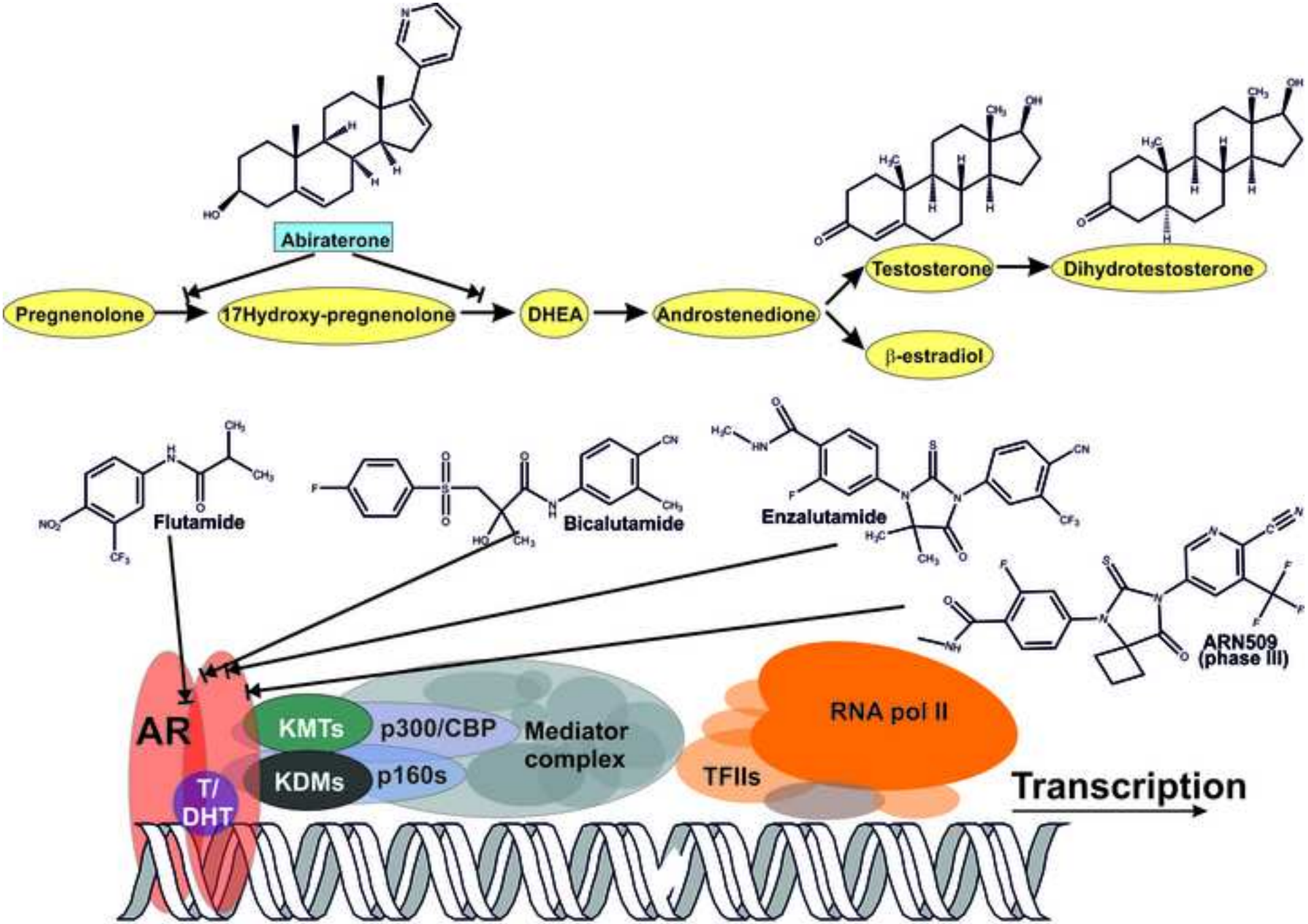

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