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Mechanism underlying the retarded nuclear translocation of androgen receptor splice variants

Ye Liu[1†], Yinyu Wang[1†], Fangfang Wang[2], Jiexue Pan[2], Jingjing Xu[1], Jingyi Li[2], Chengliang Zhou[2], Guolian Ding[1,2], Yanting Wu[1,2], Xinmei Liu[1,2], Jianzhong Sheng[2,3] & Hefeng Huang[1,2*]

[1]*The International Peace Maternity and Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200030, China;*
[2]*The Key Laboratory of Reproductive Genetics (Ministry of Education), Zhejiang University, Hangzhou 310058, China;*
[3]*Department of Pathophysiology, Zhejiang University School of Medicine, Hangzhou 310058, China*

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As shown in our previous study, two alternatively spliced androgen receptor (AR) variants, which are exclusively expressed in the granulosa cells of patients with polycystic ovary syndrome, exhibit retarded nuclear translocation compared with wild-type AR. However, researchers have not yet determined whether these abnormalities correlate with heat shock protein 90 (HSP90) and importin α (the former is a generally accepted co-chaperone of AR, and the latter is a component of classical nuclear import complexes). Here, these two variants were mainly retained in cytoplasm with HSP90 and importin α in the presence of dihydrotestosterone (DHT), and their levels in nucleus were significantly reduced, according to the immunofluorescence staining. The binding affinity of two AR variants for importin α was consistently decreased, while it was increased in WT-AR following DHT stimulation, leading to reduced nuclear import, particularly for the insertion-AR (Ins-AR). However, the binding affinities of two AR variants for HSP90 were increased in the absence of DHT compared with WT-AR, which functioned to maintain spatial structural stability, particularly for the deletion-AR (Del-AR). Therefore, the retarded nuclear translocation of two AR variants is associated with HSP90 and importin α, and the abnormal binding affinities for them play critical roles in this process.

androgen receptor splice variants, nuclear translocation, heat shock protein 90, importin α, polycystic ovary syndrome

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common reproductive endocrinopathies and is characterized by chronic anovulation or rare ovulation, hyperandrogenism and polycystic ovaries on ultrasonography (Azziz et al., 2016; Norman et al., 2007). The occurrence of PCOS in women of childbearing age is high, ranging from 5% to 10% worldwide, depending on the diagnostic criteria applied (Azziz et al., 2016; Norman et al., 2007; Rosenfield and Ehrmann, 2016). Although the underlying etiology of PCOS remains unknown (Hart, 2016), current evidence implies that androgens and the androgen receptor (AR) are important contributors to this disorder (Norman et al., 2007; Rosenfield and Ehrmann, 2016) because they play crucial roles in the pathophysiology of hyperandrogenism in PCOS.

AR, a member of the nuclear receptor superfamily of ligand-activated transcription factors, is expressed in granulosa cells (GCs) and is predominantly associated with androgen metabolism and folliculogenesis (Rosenfield and Ehrmann, 2016; Wang et al., 2015). The AR protein is organized into four functional domains encoded by eight exons: the N-terminal transactivation domain (NTD); the

†Contributed equally to this work
*Corresponding author (email: huanghefg@sjtu.edu.cn)

DNA-binding domain (DBD), which contains two zinc finger domains that are translated from exons 2 and 3; the ligand-binding domain (LBD); and the hinge region, which connects the DBD and LBD (Heemers and Tindall, 2007; Watson et al., 2015). In the DBD, the first zinc finger specifically recognizes DNA sequences known as androgen response elements (AREs), while the second facilitates AR homodimer formation and stabilizes the DNA-AR complex (Dehm and Tindall, 2011; Heemers and Tindall, 2007). Importantly, the nuclear localization signal (NLS), which recognizes characteristic nuclear import factors and participates in nuclear translocation, spans the DBD and hinge region in exons 3 and 4 (Kaku et al., 2008; Watson et al., 2015). Alternative splice variants (ASVs) are created during pre-mRNA modification (Liu et al., 2014; Savage, 2015) and have previously been investigated in pathologies ranging from prostate cancer (Shafi et al., 2013; Watson et al., 2015) to androgen insensitivity syndromes (Dehm and Tindall, 2011; Hughes et al., 2012). Our team has recently identified two ASVs of AR (Wang et al., 2015) that are exclusively expressed in the luteinized human GCs of women with PCOS and documented the functional consequences of these ASVs in terms of hyperandrogenism and ovarian folliculogenesis. One ASV contains an insertion of 69 base pairs between exons 2 and 3 (Ins-AR), and the other skips exon 3 (Del-AR), indicating that these changes alter the secondary and three-dimensional structures of the DBD, particularly the second zinc finger (Wang et al., 2015). Because the binding of AR to its ligand leads to translocation to the nucleus (Shafi et al., 2013), we observed abnormal nuclear translocation and impaired transcription factor function of these GC-specific AR ASVs, consistent with previous studies (Jagla et al., 2007; Wadosky and Koochekpour, 2017; Zhu et al., 1997).

Because the formation of a productive DNA-AR complex requires the functional and structural interaction of AR with its co-regulators, several proteins that interact with AR have been identified to date (Heemers and Tindall, 2007), including heat shock proteins (HSPs) and importins. HSPs are a type of chaperone that primarily reside in the cytoplasmic compartment, where they form complexes with AR to modify its stability and maintain its subcellular localization in the unliganded state (Azad et al., 2015; Ischia and So, 2013). These macro-molecules are named according to their approximate molecular weight (kD), including HSP90 and other proteins (Ischia and So, 2013). HSP90, a main member of the HSP family, is involved in the folding, stabilization and translocation of AR, a known HSP90 client protein (Chen et al., 2016; Ischia and So, 2013; Kirschke et al., 2014). In the early stages of the AR activation process, the AR LBD interacts transiently with these molecular chaperones, giving rise to a balance in which AR is maintained in an overall high-affinity ligand-binding state (Azad et al., 2015; Heemers and Tindall, 2007). Upon stimulation with androgens, HSP90 disassociates, and AR undergoes a sequence of conformational changes and is translocated to the nucleus due to unmasking of the NLS (Heemers and Tindall, 2007; Shafi et al., 2013). In addition to the aberrant nuclear translocation caused by HSPs, nuclear import factors are also thought to participate in the mechanism regulating AR activity. The two most important import factors involved in the classical nuclear protein import machinery are importin α and importin β (also called karyopherin-α and karyopherin-β) (Pemberton and Paschal, 2005). Importin α functions by directly binding to specific NLS motifs (Cutress et al., 2008; Sun et al., 2017) on the AR proteins in the cytoplasm, while importin β serves as a coactivator that mediates translocation (Ni et al., 2013). The importin α-importin β complex then moves through the nuclear pore complexes (NPCs) to enter the nucleus, and thereafter, both importins are returned to the cytoplasm for a new round of protein import (Cutress et al., 2008; Ni et al., 2013).

Although we previously reported the retarded nuclear translocation of AR ASVs, the question of whether HSPs or nuclear import factors are associated with this process has not yet been assessed. Therefore, our previous study fostered a greater interest in investigating the mechanism underlying the abnormal nuclear translocation of Ins-AR and Del-AR.

RESULTS

The nuclear translocation of ASVs is reduced compared with WT-AR following DHT treatment

The nuclear translocation of WT-AR and the ASVs in transfected KGN cells was determined by confocal immunofluorescence microscopy. Both WT-AR and the ASVs remained in the cytoplasm in the absence of dihydrotestosterone (DHT) (Figure 1A). However, the treatment of WT-AR-overexpressing KGN cells with DHT dramatically increased nuclear translocation compared to cells grown in the absence of DHT, whereas both Ins-AR and Del-AR showed reduced nuclear translocation; more ASVs than WT-AR were retained in the cytoplasm (Figure 1B). We separately measured the fluorescence signal in the cytoplasm and nucleus to quantify the relative fluorescence intensity in different cellular regions. Then, we calculated the ratio of cytoplasmic to total fluorescence intensity in the absence and presence of DHT, as shown in Figure 1C. The fluorescence intensity of WT-AR in the cytoplasm (blue bar) was significantly reduced by the DHT treatment. However, the fluorescence intensity of Ins-AR (red bar) and Del-AR (green bar) in the cytoplasm was slightly, but not significantly, reduced. In addition, we also calculated the ratio of nuclear to total fluorescence intensity in the presence or absence of DHT treatment, as shown in Figure 1D. Consistently, the intensity of WT-AR was remarkably increased

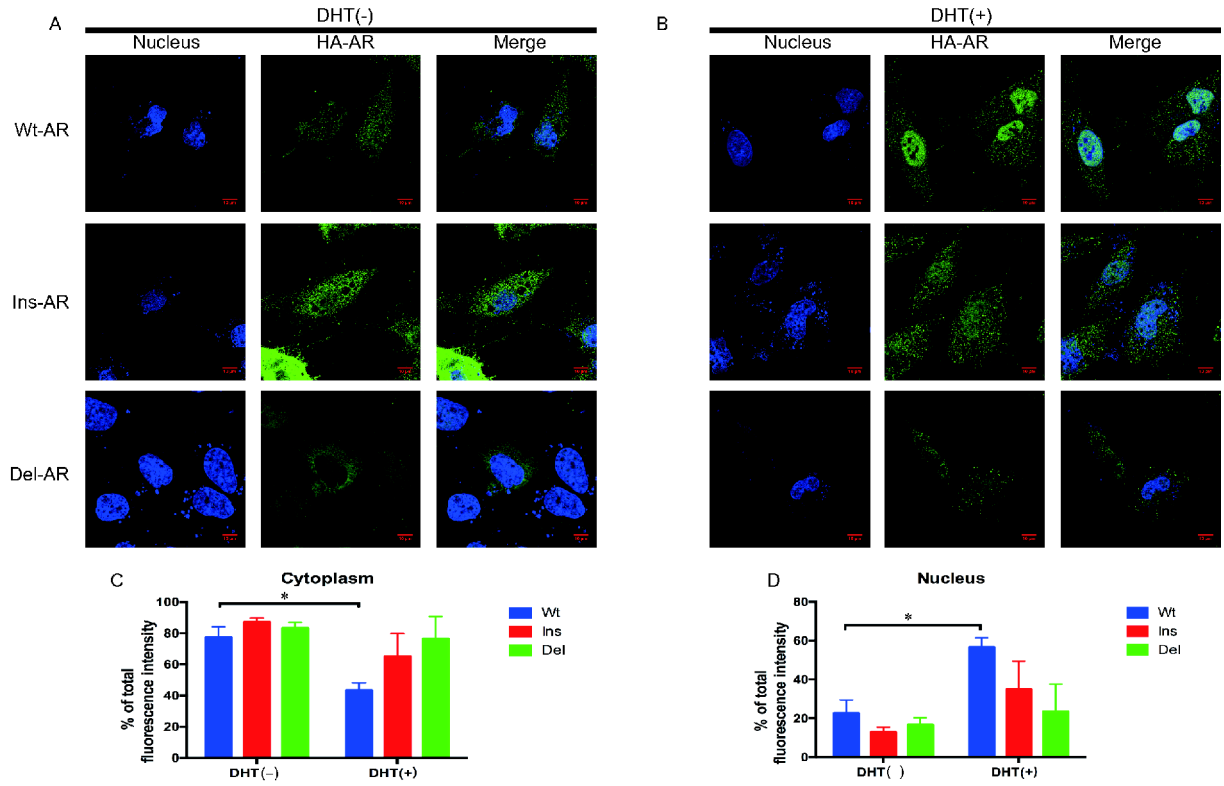

Figure 1 Distributions of WT-AR and the ASVs (Ins-AR and Del-AR) between the cytoplasm and nucleus of KGN cells in response to the DHT treatment. A, WT-AR and the ASVs were transfected into KGN cells, and their localization in the cytoplasm and nucleus was observed in the absence of androgen. B, KGN cells were exposed to DHT (10^{-7} mol L^{-1} for 2 h), and their localization in the cytoplasm and nucleus was also observed. Cellular localization was determined by confocal immunofluorescence microscopy at 63× magnification, and immunofluorescence staining revealed the distinct nuclear translocation of the ASVs, confirming the antibody specificity. Bar, 10 μm. C and D, Fluorescence signals of HA-AR in the cytoplasm and nucleus were quantified separately using ImageJ software. Quantities are shown as the ratio of cytoplasmic or nuclear signal to the total fluorescence intensity. Values are presented as means±SE. N=9; *, P<0.05.

after DHT stimulation, while the two variants presented a decreased response to DHT compared to WT-AR. A time course examination of the subcellular localization of WT-AR and the ASVs was performed to precisely assess nuclear translocation of the ASVs. WT-AR was rapidly translocated to the nucleus within 30 min of DHT exposure, as shown in Figure S1A in Supporting Information. The ASVs showed delayed nuclear import at 30 min and only partial nuclear localization was observed for ASVs at 48 h, as shown in Figure S1B and C in Supporting Information. This finding confirmed the dysfunctional nuclear translocation of ASVs because these two splice variants displayed reduced nuclear localization upon androgen exposure. Given the critical roles of HSPs, mainly HSP90, in nuclear transport and importins in nuclear import, we then investigated the total levels of these proteins by western blot and examined any alterations occurring in response to DHT stimulation.

Exogenous ARs (WT-AR, Ins-AR and Del-AR) do not influence the expression of HSP90 and importin α

First, we transfected cells with AR and the ASVs and detected their protein levels to confirm successful overexpression. According to PCR and western blot, the total mRNA and protein levels of exogenous WT-AR and the ASVs in the overexpressing 293T cells were remarkably increased compared with the negative control cells. As shown in Figure 2B and C, the Ins-AR protein was indeed expressed at a lower level than WT-AR and Del-AR; meanwhile, the mRNA levels shown in Figure 2A were not significantly different between the groups, suggesting some obstacles in the translation of the Ins-AR protein may exist. Upon androgen stimulation, levels of the WT-AR and ASV proteins were slightly and significantly increased (Figure 2B and C). In addition, the total levels of the HSP90 and importin α mRNAs and proteins were investigated. The alterations in the levels of these mRNA and proteins in response to the DHT treatment were small, and no difference was observed between transfected cells and negative control cells in the absence of androgen, suggesting that exogenous AR (WT-AR, Ins-AR and Del-AR) did not influence the expression of HSP90 and importin α and that the overexpressing 293T cells represented a stable cell model for use in subsequent experiments (Figure 2). Because both HSP90

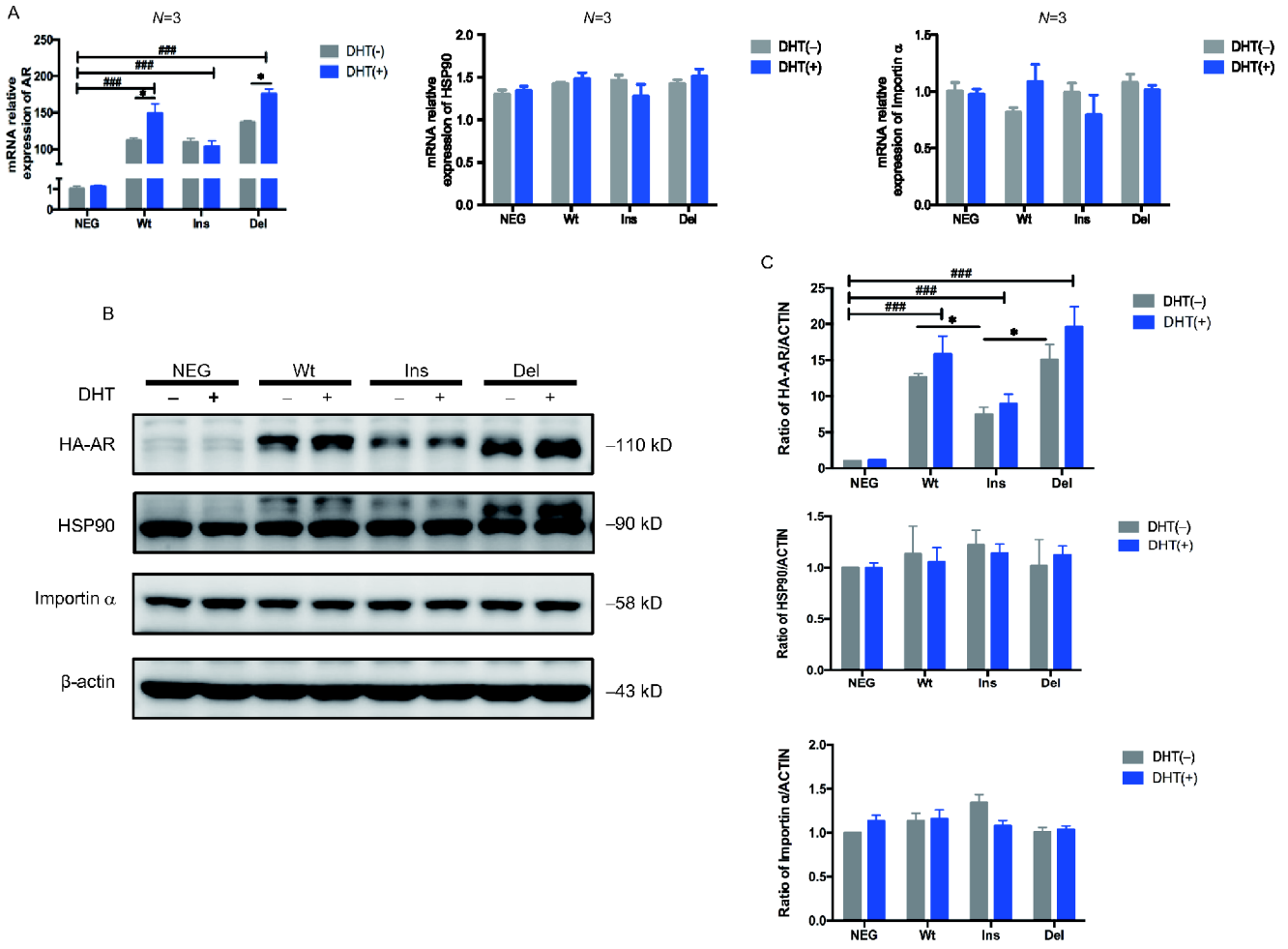

Figure 2 Total levels of the WT-AR and ASV mRNAs and proteins (Ins-AR or Del-AR), HSP90 and importin α in the absence or presence of DHT. A, *WT-AR* and the *ASVs* were overexpressed in 293T cells, and then the total mRNA was extracted to compare changes in the levels of the *AR* (WT-AR, Ins-AR or Del-AR), *HSP90* and *importin α* mRNAs in response to DHT (10^{-7} mol L^{-1} for 2 h) and in the negative control cells. B, WT-AR and the ASVs were overexpressed in 293T cells, and then the total proteins were extracted to compare changes in the levels of AR (WT-AR, Ins-AR or Del-AR), HSP90 and importin α in response to DHT (10^{-7} mol L^{-1} for 2 h) and in the negative control cells. C, Total levels of the AR, HSP90 and importin α proteins were quantified using Quantity One software, and the results are shown as ratios relative to the β-actin level.

and importin α participate in AR shuttling from the cytoplasm to the nucleus, we were interested in investigating their individual distributions.

ASVs attenuate the nuclear translocation of importin α, with less of an effect on HSP90

Because we did not detect apparent differences in the total levels of the HSP90 and importin α proteins in overexpressing 293T cells, we decided to determine the subcellular distributions of HSP90 and importin α between the cytoplasm and nucleus, as well as their co-localization with WT-AR and the ASVs by immunofluorescence staining. Similar to our initial experiments, the ASVs presented a decreased response to the DHT treatment and were mainly retained in the cytoplasm; in contrast, WT-AR exhibited normal nuclear translocation, and the amount of WT-AR in the nuclear fraction increased after DHT stimulation. Additionally, both WT-AR and ASVs co-localized with HSP90 (Figure 3A) and importin α (Figure 3B) in the absence and presence of DHT, indicating that ASVs also interacted with these two molecules, despite their altered structural conformations. Then, we compared the fluorescence intensity of HSP90 and importin α between the cytoplasm and nucleus, as shown in Figure 3C–F. In the absence of DHT, HSP90 was mainly located in the cytoplasm along with WT-AR and ASVs, but a remarkable translocation into the nucleus occurred after DHT stimulation (Figure 3C and D), illustrating that HSP90 also participated in nuclear import. The subcellular localization of WT-AR and importin α in the absence of androgen was predominantly cytoplasmic, but these proteins were rapidly imported into the nucleus upon androgen

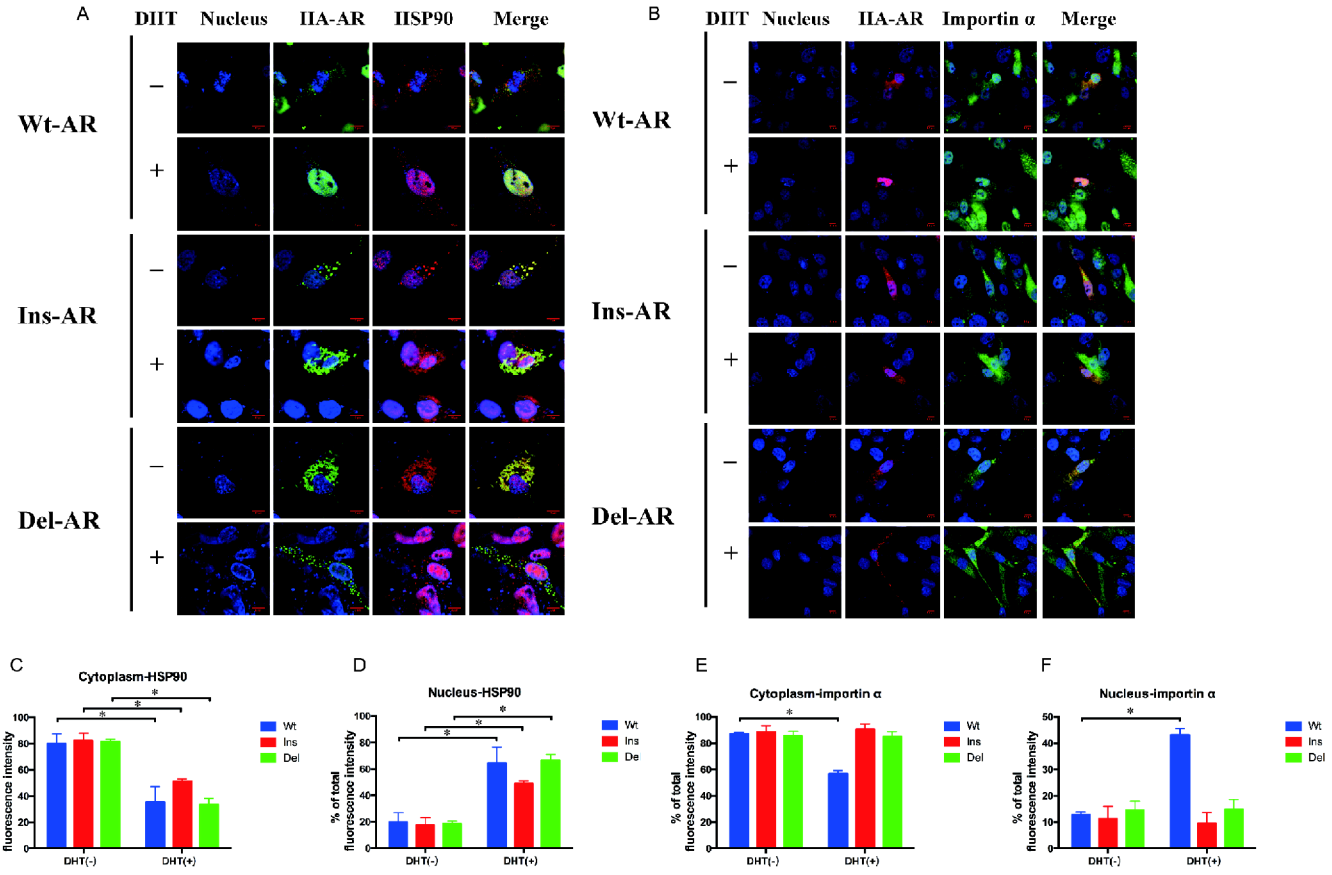

Figure 3 Subcellular distributions of HSP90 and importin α between the cytoplasm and nucleus and their co-localization with WT-AR and the ASVs in response to the DHT treatment. A, WT-AR and the ASVs (Ins-AR and Del-AR) were transfected into KGN cells, and the subcellular localization of HSP90 was detected in the absence or presence of DHT (10^{-7} mol L^{-1} for 2 h) by confocal immunofluorescence microscopy at 63× magnification. B, The co-localization of importin α was investigated in AR (WT-AR, Ins-AR and Del-AR)-overexpressing cells. Because the primary antibodies against HSP90 and importin α were developed in different species, the secondary antibodies were assigned to the wavelengths 488 (green) and 594 (red). Bar, 10 μm. C–F, The fluorescence signals of HSP90 and importin α in the cytoplasm and nucleus were quantified separately using ImageJ software. Quantities are shown as the ratio of the cytoplasmic or nuclear signal to the total fluorescence intensity. Values are presented as means±SE. *, $P<0.05$.

treatment. Although the ASVs were also located in the cytoplasm along with importin α, the DHT treatment did not induce nuclear translocation to the same extent as observed for WT-AR (Figure 3E and F). Based on these results, WT-AR undergoes nuclear translocation with the help of HSP90 and importin α, whereas Ins-AR and Del-AR show reduced nuclear transport. In addition, HSP90 participates in nuclear translocation in WT-AR- and ASV-transfected cells stimulated with androgen, while importin α exhibits impaired nuclear translocation in ASV-transfected cells, indicating that the differences in the binding affinities of ASVs for HSP90 and importin α may reduce nuclear translocation.

HSP90 and importin α exhibit reduced binding affinity for ASVs compared to WT-AR

Because HSP90 exhibited normal nuclear translocation and importin α was significantly retained in the cytoplasm after DHT treatment in cells transfected with the two AR variants, we performed co-immunoprecipitations to analyze the ability of HSP90 and importin α to bind to the ASVs. HSP90 and importin α bound to WT-AR and the ASVs under both androgen-free and androgen-enriched conditions (Figure 4A). On one hand, the binding affinity of HSP90 for the ASVs was greater than for WT-AR in the absence of DHT; in particular, Del-AR-overexpressing cells presented a nearly two-fold increase in binding affinity compared with Ins-AR-expressing cells. However, these two ASVs exhibited a dramatic reduction in binding affinity for HSP90 upon androgen stimulation; Del-AR exhibited a greater decrease than Ins-AR, and this difference was consistently statistically significant, whereas WT-AR showed no obvious alteration (Figure 4B). On the other hand, the binding affinity of importin α for the ASVs was predominantly increased, particularly for Ins-AR, which showed a three-fold higher affinity than WT-AR. However, both Ins-AR and Del-AR showed decreased binding affinities for importin α in cells treated with DHT, particularly Ins-AR. In contrast, the binding af-

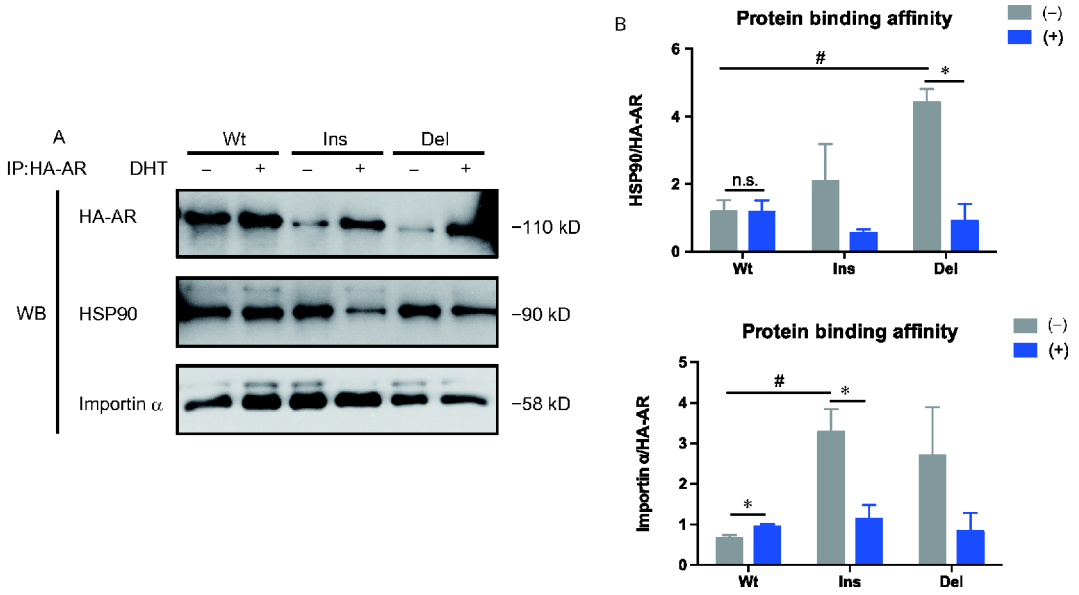

Figure 4 Binding affinities of HSP90 and importin α for WT-AR and the ASVs (Ins-AR or Del-AR), according to the co-immunoprecipitation results. A, The 293T cells were transfected with WT-AR and the ASVs (Ins-AR and Del-AR) to assess the binding affinities of HSP90 and importin α by co-immunoprecipitation in cells that had been treated with 10^{-7} mol L^{-1} DHT for 2 h. B, The binding affinities of both ASVs for HSP90 were markedly decreased in cells treated with DHT, particularly Del-AR, but little difference was observed in WT-AR-expressing cells. Similarly, the binding affinity of ASVs for importin α was dramatically reduced following DHT stimulation, particularly Ins-AR, whereas WT-AR exhibited a slight increase in binding affinity. Bars indicate the ratio relative to HA-AR, and values are presented as means±SE. * and #, $P<0.05$.

finity of importin α for WT-AR appeared to be increased to a greater extent than HSP90 (Figure 4B). Overall, HSP90 and importin α presented reduced binding affinities for ASVs compared to WT-AR, and both ASVs showed abnormal nuclear translocation. HSP90 is a key molecular chaperone that maintains the structural stability of AR and transports it into NPCs, while importin α plays a crucial role in nuclear import. Thus, these two molecules perform their main biological functions in the cytoplasm and nucleus, respectively. Therefore, we next separately extracted cytoplasmic and nuclear proteins and performed western blots to further investigate the alterations in HSP90 and importin α functions in the presence of ASVs.

ASVs disrupt the nuclear and cytoplasmic distributions of importin α following DHT exposure, with less of an effect on HSP90

We isolated the total proteins from WT-AR- and ASVs-overexpressing 293T cells and prepared cytoplasmic and nuclear protein fractions; we then performed western blots to investigate the expression of WT-AR, the ASVs, HSP90 and importin α (Figure 5A and C). In the cytoplasm, WT-AR levels were similar to the ASVs in the absence of DHT. Upon androgen stimulation, WT-AR levels were decreased in the cytoplasm, apparently due to nuclear translocation, while the levels of both Ins-AR and Del-AR increased, implying that nuclear import was impaired. In addition, the levels of the HSP90 and importin α proteins did not show abnormal alterations compared with negative control cells. The levels of HSP90 were significantly decreased in cells overexpressing WT-AR and the ASVs following androgen treatment, whereas importin α levels remained unchanged (Figure 5B). In the nucleus, the levels of the WT-AR and ASV proteins were increased following androgen treatment; in particular, the WT-AR-transfected cells exhibited a remarkable elevation in the nuclear levels of the AR protein compared with the ASVs. Thus, WT-AR showed a normal reaction towards androgens and was transported into the nucleus. The amount of the HSP90 protein was also increased in all three types of transfected cells in response to DHT treatment, similar to negative control cells. Moreover, importin α levels were increased in WT-AR-overexpressing cells in the presence of DHT, whereas its levels were decreased in both Ins-AR- and Del-AR-expressing cells after DHT exposure, particularly in Ins-AR-transfected cells (Figure 5D). In summary, cytoplasmic WT-AR levels decreased and nuclear WT-AR levels increased in response to the DHT treatment, accompanied by elevated levels of HSP90 and importin α. Cytoplasmic Ins-AR levels increased more dramatically than nuclear Ins-AR levels, and nuclear importin α levels were apparently reduced after androgen stimulation. The alterations in levels of the Del-AR protein were similar to Ins-AR, but the differences were not significant. These findings are consistent with the results of the co-localization experiment.

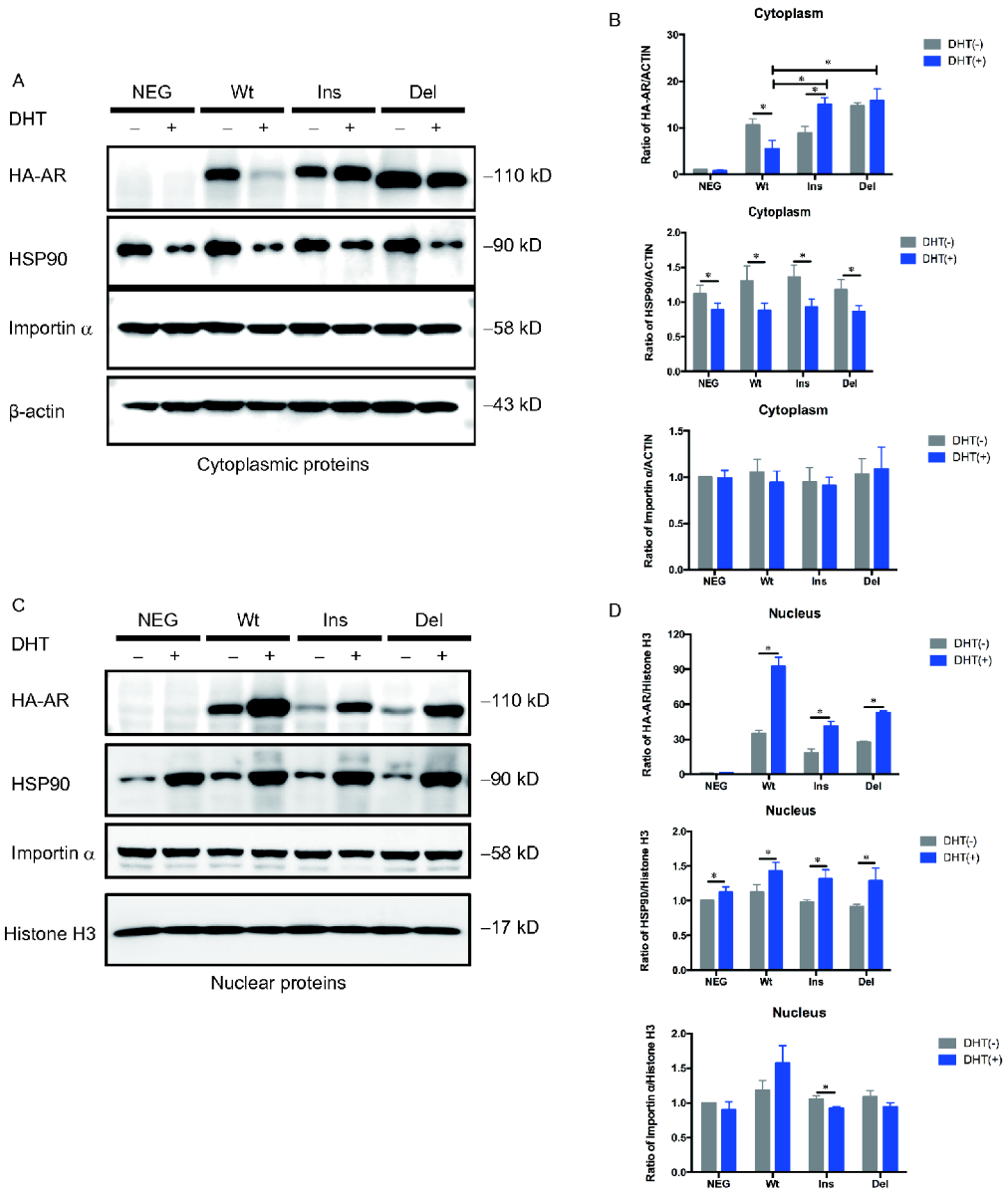

Figure 5 Cytoplasmic and nuclear levels of the WT-AR, ASV (Ins-AR or Del-AR), HSP90 and importin α proteins in the absence or presence of DHT. A and C, The 293T cells overexpressing WT-AR and the ASVs were fractionated into cytoplasmic proteins (A) and nuclear proteins (C) after culture in the absence or presence of DHT (10^{-7} mol L^{-1} for 2 h). The fractions were subjected to western blot analysis. β-Actin and histone H3 were used as housekeeping proteins. B and D, The cytoplasmic and nuclear levels of the AR, HSP90 and importin α proteins were quantified using Quantity One software, and the results shown here are representative of three independent experiments. Quantities are shown as the ratio to levels of the β-actin or histone H3 protein, respectively. Values are presented as means±SE. *, $P<0.05$.

DISCUSSION

In the present study, we investigated the potential mechanism underlying the abnormal nuclear translocation of two ASVs that were initially discovered by our team in the GCs of patients with PCOS. In addition, we characterized the effects of HSP90 and importin α on the pathogenesis of retarded nuclear translocation.

Because HSPs are chaperones that stabilize AR and promote its binding to androgens (Azad et al., 2015; Chen et al., 2016), we examined the levels of the HSP90 protein in the total, cytoplasmic and nuclear protein fractions, as well as its binding affinity for WT-AR and the ASVs. The binding affinity of HSP90 for Del-AR was significantly increased in the absence of androgens, and we postulate that HSP90 plays a more crucial role in the abnormal nuclear translocation of Del-AR than Ins-AR. In the unliganded state, HSP90 binds to the AR LBD (Heemers and Tindall, 2007), which is encoded by exons 4 to 8, in the cytoplasm. The spatial structure of LBD is more substantially influenced by the deletion of exon 3 than by the insertion of 69 base pairs between exons 2 and 3, similar to the findings reported by Helsen et al.

(Helsen et al., 2012), indicating that the mutation residing in the second zinc finger of the DBD reduces the ligand-binding affinity (Huang et al., 2013). In addition, AR proteolysis is predominantly regulated by the HSP90-based chaperone machinery, and HSP90 inhibits AR ubiquitination (Azad et al., 2015; Pratt et al., 2015). Therefore, more HSP90 must interact with Del-AR than with Ins-AR to maintain a stable configuration with a high affinity for androgen ligands. Although the total levels of the HSP90 protein were not significantly different between the groups, the amount of the high-affinity form of HSP90 is more crucial than the total cellular levels of HSP90 for maintaining AR function and survival (Georget et al., 2002). Similar results were observed in cancer cells with high loads of mutated AR (Ischia and So, 2013). HSP90 also participates in AR import via the NPCs upon androgen exposure (Galigniana et al., 2010; Grossmann et al., 2012). The binding affinity of HSP90 for Del-AR was markedly reduced in the presence of DHT, and a non-significant reduction in importin α binding was observed compared to Ins-AR, suggesting that more Del-AR is imported into the nucleus than Ins-AR because the dissociation of HSP90 from AR facilitates greater exposure of the NLS (Shafi et al., 2013; Tao and Zheng, 2011), and efficient nuclear shuttling of AR depends on its NLS (Grossmann et al., 2012; Marfori et al., 2011). Based on these results, Del-AR is more unstable than Ins-AR and thus must bind more HSP90 in the androgen-free state. However, HSP90 is more easily dissociated from AR, subsequently increasing nuclear import. This hypothesis might be a possible explanation for why patients carrying Ins-AR tend to experience more severe symptoms than patients carrying Del-AR, such as higher serum androgen levels and greater dysfunction in follicle development (Wang et al., 2015).

In addition to the role of HSP90 in transporting AR, importin α contributes to AR import into the nucleus (Marfori et al., 2011). Therefore, in the second part of our study, we examined the potential role of importin α. According to the co-immunoprecipitation results, both Ins-AR and Del-AR, particularly Ins-AR, exhibited higher binding affinities for importin α in the absence of DHT than WT-AR, but both manifested a remarkable reduction in binding affinity upon androgen exposure. Hence, we hypothesize that importin α may be the predominant reason for the abnormal nuclear translocation of Ins-AR. Importin α binds to the AR NLS (Forwood et al., 2001), which is encoded by exons 3 and 4. Although both ASVs exhibit an altered structure of the second zinc finger in exon 3 that interferes with the conformation of the DBD, thereby indirectly preventing binding of importin α, the lack of exon 3 in Del-AR leads to an intact NLS sequence but the retention of partial binding affinity to importin α because AR exon 4 is more important for the well-characterized NLS structure and provides the major binding site for importin α (Chan et al., 2012; Dehm and Tindall, 2011; Sprenger and Plymate, 2014). In contrast, the disturbance of the spatial conformation caused by the insertion between exons 2 and 3 in Ins-AR substantially reduces the binding affinity for importin α (Jagla et al., 2007). This potential mechanism probably relies on the structure of the AR DBD, which is critical for the activity of the AR NLS (Shostak and Yamamoto, 2005; Yang et al., 2011) and is consistent with the findings reported by Ni et al. (Ni et al., 2013). These authors examined importin 7, which causes cytoplasmic retention of AR by binding to the NLS. Consistent with these findings, the nuclear level of the importin α protein was dramatically reduced by the androgen treatment, indicating that a smaller amount of importin α transports Ins-AR into the nucleus than Del-AR, and nuclear import was mostly abrogated.

However, our study has some limitations. First, because of the important roles of HSP90 in inducing AR nuclear translocation, we have focused on this chaperone in our experiments instead of other members of the HSP family, such as HSP70 and HSP40 (Azad et al., 2015; Heemers and Tindall, 2007; Pratt et al., 2015). Second, many other co-chaperones, such as Hop (Alvira et al., 2014; Tao and Zheng, 2011) and p23 (Fang et al., 2006; Tao and Zheng, 2011), regulate HSP90 activity through various post-translational modifications, including phosphorylation, acetylation, oxidation, S-nitrosylation and ubiquitination (Azad et al., 2015; Jiménez-Canino et al., 2016). We have simply examined the binding affinity and levels of the HSP90 protein but have not analyzed the epigenetic modifications of HSP90. Third, importin-α-importin-β complexes participate in the most classical nuclear import pathway. However, based on recent studies, numerous proteins perform nuclear import functions by binding directly to importin β (Forwood et al., 2001; Marfori et al., 2011; Pemberton and Paschal, 2005) without binding to importin α. These two ASVs may initiate nuclear import through importin β alone and participate in fewer interactions with importin α. Finally, other molecules probably participate in regulating the intracellular movement of AR, such as the actin-binding protein filamin (Ozanne et al., 2000) and β-catenin (Kaku et al., 2008; Pawlowski et al., 2002). Hence, further studies are needed to evaluate the possible effects of these proteins and pathways to determine the specific mechanism underlying the retarded nuclear translocation of Ins-AR and Del-AR.

In summary, the two ASVs that have primarily been identified in GCs from patients with PCOS by our team exhibited abnormal nuclear translocation compared with WT-AR. In addition, the disorder relates to two different molecules: HSP90, which plays a pivotal role in the altered transport of Del-AR, and importin α, which contributes to the retarded nuclear import of Ins-AR. In addition, patients who carry Del-AR present milder symptoms than patients carrying Ins-AR, according to a previous clinical investigation

(Wang et al., 2015), and the reason may involve a greater reduction in HSP90 levels and the increased nuclear translocation of Del-AR. Thus, we presume that studies aiming to understand the abnormal nuclear translocation of these ASVs, which results in ovarian dysfunction in patients with PCOS, will assist in the development of ASV-targeted treatments as a novel therapeutic approach to benefit patients with PCOS.

MATERIALS AND METHODS

Human granulosa tumor cell line (KGN) and HEK 293T cell line

The human ovarian granulosa-like tumor cell line KGN and the HEK 293T cell line were obtained from Riken Gene Bank (Tsukuba, Japan) and American Type Culture Collection (USA), respectively. KGN cells were grown in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco 11039-021; Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Australia Origin, 10099141). KGN cells participate in most physiological ovarian activities and, consequently, were used in most of our experiments. In addition, 293T cells were grown in phenol red-free DMEM/high glucose (Gibco 10569-010; Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum. Both KGN and 293T cells were cultivated in an incubator at 37°C with a 5% (v/v) CO_2 atmosphere. Cells were stored in liquid nitrogen for future use.

Transient transfection

The 293T cells and KGN cells were maintained as described above. For transient transfection experiments, 5×10^5 cells were seeded on 10 cm dishes and coverslips, respectively. After 24 h, 1 μg mL^{-1} of plasmids encoding AR ASVs were transiently transfected into cells using X-tremeGENE HP transfection reagent (Roche, USA) according to the manufacturer's protocol. After 48 h, the transfected cells were treated with 10^{-7} mol L^{-1} DHT (Sigma-Aldrich, USA) or an equivalent amount of vehicle (ethanol) in serum-free media for 2 h. DHT binds to AR with higher affinity than endogenous androgens, and its biological activity exceeds testosterone. The treated cells were collected and preserved for further experiments.

Immunofluorescence staining

Cells were seeded on coverslips 1 day before the experiments. After two rinses with cold PBS, the cells were fixed with a 4% paraformaldehyde solution, followed by permeabilization with 0.1% Triton X-100/PBS (Sigma-Aldrich). KGN cells were sequentially incubated with a blocking solution (3% BSA/PBS), primary antibody, and secondary antibody with DAPI. Antibodies against the HA tag (1:200, ab9110, Abcam, USA; 1:200, E0020, Anbo, USA), HSP90 (1:200, ab13492, Abcam), and importin α (1:200, ab70160, Abcam) were used as primary antibodies. Alexa Fluor 488-conjugated goat anti-rabbit (1:200, A-11034, Invitrogen, USA) and Alexa Fluor 594-conjugated goat anti-mouse (1:200, A-11020, Invitrogen) antibodies were used as secondary antibodies. After an incubation in the secondary antibody solutions, the cells were washed with cold PBS three times, and the coverslips with attached cells were mounted. Cells were imaged using a laser-scanning confocal microscope fitted with 10× and 63× objective lenses (Zeiss 800, LSM 510 Meta, Austria). After the coverslips were mounted, the slides were stored in a dark, cold room until images were captured.

Co-immunoprecipitation

For co-immunoprecipitation experiments, 293T cells plated on 10 cm dishes were transiently transfected with plasmids (HA-tagged WT or Ins-AR or Del-AR plasmids) (GeneCopoeia, USA), and exposed to the same treatment conditions in the subsequent experiments. At 48 h post-transfection, the interactions between AR and HSP90 and importin α were assessed by co-immunoprecipitation in the presence of 10^{-7} mol L^{-1} DHT or an equivalent amount of vehicle (ethanol) for 2 h. Cells were washed three times with cold PBS and lysed in cold lysis buffer (50 mmol L^{-1} Tris-HCl, pH 7.5; 100 mmol L^{-1} NaCl; 1 mmol L^{-1} EDTA; 0.5% Nonidet P-40) supplemented with protease inhibitors. Equal amounts of cell lysates were incubated with the anti-HA antibody (ab9110, Abcam) overnight at 4°C. Immunocomplexes were collected with Protein A/G PLUS-Agarose (sc-2003, Santa Cruz, USA) and tested for the presence of the target proteins by western blot.

Western blot

Samples were lysed with RIPA buffer containing 1% PMSF (Beyotime, Shanghai) for 30 min on ice. Whole-cell lysates were obtained by centrifugation at 14,000×*g* for 15 min at 4°C. After the protein concentration was determined using the bicinchoninic acid (BCA) method (23225, Thermo Scientific Pierce, USA), whole-cell lysates and immunocomplexes were separated on a 10% (v/v) sodium dodecyl sulfate-polyacrylamide gels, transferred to PVDF membranes, and exposed to mouse anti-HSP90 (1:3000, ab13492, Abcam), rabbit anti-importin α (1:5000, ab70160, Abcam), mouse anti-β-actin (66009, Proteintech, USA) and rabbit anti-histone H3 (1:3000, ab1791, Abcam) antibodies overnight at 4°C, followed by exposure to diluted goat anti-

rabbit (1:5000, #7074, CST) or mouse secondary antibodies (1:5000, #7076, CST) for 2 h at room temperature. Signals were detected with an enhanced chemiluminescence detection reagent (PE0010, Solarbio, USA). β-Actin is a major component of the cytoskeleton and therefore served as the internal control. The integrated densities of the bands were quantified using Quantity One 4.6.8 software (Bio-Rad, USA).

Co-localization studies

The effects of HSP90 and importin α on the subcellular localization of the AR and ASVs in cells treated with DHT (10^{-7} mol L^{-1}) or an equivalent vehicle (ethanol) were examined using immunofluorescence staining for HA-tagged AR. The detailed protocols are described above. Some critical procedures differed from the immunofluorescence staining protocol as described below. First, either a mixture of rabbit anti-HA tag (1:200 dilution) with mouse anti-HSP90 (1:50) in antibody diluent or mouse anti-HA tag (1:200 dilution) with rabbit anti-importin α (1:200) in antibody diluent was added to the cells and incubated at 4°C overnight. Then, Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 594-conjugated goat anti-mouse secondary antibody solutions were prepared at 1:200 dilutions in the blocking solution and added to the cells. The cells were incubated in the dark at 37°C for 2 h.

Preparation of protein extracts (nuclear and cytoplasmic fractionation)

Cells were collected and counted after 48 h of transfection. The cells were equally divided into two parts, one for total protein detection, and the other partitioned into cytoplasmic and nuclear fractions using a nuclear and cytoplasmic protein extraction kit (Beyotime, P0027), according to the manufacturer's instructions. Protein quantification was performed as described above.

Statistical analysis

Data are presented as means±SE (n is the number of tissue preparations, cells or separate experiments, as indicated in the figure legends). Statistical analyses were performed using an unpaired 2-tailed Student's t-test or equivalent nonparametric tests (SPSS version 15.0). A P value <0.05 was considered statistically significant.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Time course of changes in the distributions of WT-AR and the ASVs (Ins-AR and Del-AR) between the cytoplasm and nucleus in KGN cells in response to DHT treatment.

Figure S2 The total AR ASVs, HSP90 and importin α proteins in cells were partitioned into cytoplasmic and nuclear fractions and used as input for Co-IP experiments.

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