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Department of Reproduction and Development, Erasmus MC (H.Y.W., K.L.P., J.H., M.v.L., C.A.B., J.A.G., A.O.B.); Department of Pathology, Josephine Nefkens Institute, Erasmus MC (M.E.v.R., H.J.D, M.M., D.J.v.d.W., J.T.); Department of Clinical Genetics, Erasmus MC (D.D., W.J.K.); Department of Urology, Erasmus MC-Sophia (K.P.W.); Department of Pediatrics, Division of Endocrinology, Erasmus MC-Sophia (S.L.S.D.), Rotterdam, the Netherlands.

Running title: Novel activating mutation F826L in the human AR

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All correspondence to:
Hao Yun Wong
Department of Reproduction and Development
Erasmus MC, Room Ee 902
P.O. Box 2040
3000 CA Rotterdam, the Netherlands
Phone: (+31)-10-7043574
Fax: (+31)-10-7044736
E-mail: h.wong@erasmusmc.nl

Abbreviations: aa, amino acid; AD, activation domain; AF2, activation function 2; DHT, 5α-dihydrotestosterone; DSD, disorders of sex development; GFP, green fluorescent protein; GSF, genital skin fibroblast; LBD, ligand binding domain; LBP, ligand binding pocket; N-CoR, nuclear receptor co-repressor; NC-TDI, NH_2-/COOH-terminal domain interaction; NTD, N-terminal transactivation domain; T, testosterone; TIF2, transcriptional intermediary factor 2; wt, wild-type.

Abstract

A novel mutation F826L located within the ligand binding domain (LBD) of the human androgen receptor (AR) was investigated. This mutation was found in a boy with severe penoscrotal hypospadias (classified as 46,XY DSD). The AR mutant F826L appeared to be indistinguishable from the wild-type AR, with respect to ligand binding affinity, transcriptional activation of MMTV-luciferase and ARE_2-TATA-luciferase reporter genes, protein level in genital skin fibroblasts (GSFs), and sub-cellular distribution in transfected cells. However, an at least two-fold higher NH_2-/COOH-terminal domain interaction was found in luciferase and GST pull-down assays. A two-fold increase was also observed for TIF2 (transcription intermediary factor 2) co-activation of the AR F826L COOH-terminal domain. This increase could not be explained by a higher stability of the mutant protein, which was within wild-type range. Repression of transactivation by the nuclear receptor co-repressor (N-CoR) was not affected by the AR F826L mutation. The observed properties of AR F826L would be in agreement with an increased activity rather than with a partial defective AR transcriptional activation. It is concluded that the penoscrotal hypospadias in the present case is caused by an as yet unknown mechanism, which still may involve the mutant AR.

Introduction

The androgen receptor (AR) belongs to the superfamily of nuclear receptors (Mangelsdorf et al., 1995; Beato et al., 1995) and is categorized in the subfamily of ligand-inducible steroid hormone receptors. Like the other steroid hormone receptors, the AR consists of an NH_2-terminal transactivation domain (NTD; amino acids 1-557), a DNA binding domain (DBD; aa 558-623), followed by a flexible hinge region (aa 624-670) and a ligand binding domain (LBD; aa 671-919) (Lubahn et al., 1988).

The NTD and the LBD account for the transcriptional activity of the AR (Brinkmann et al., 1999). The NTD is involved in transactivation via the ligand dependent activation function 1 (AF1) region, which consists of aa 51-217 (Jenster et al., 1991). The LBD harbours the AF2, which is involved in ligand-dependent transactivation, and it also takes part in functional interaction with co-factors (Moras and Gronemeyer, 1998; Jenster et al., 1995). Upon ligand binding, a large group of co-factors can interact via the AF2, which is identified in helix 12 between residues 893 and 900 (Wurtz et al., 1996). For example, TIF2 co-activation occurs when TIF2 binds to this AF2 activation domain (AD) core via its LXXLL leucine motif and thereby enhances AR transactivation (Slagsvold et al., 2000; Heery et al., 1997; Berrevoets et al., 1998; Bevan et al., 1999). Besides co-activators, also co-repressors such as nuclear receptor co-repressor (N-CoR) can bind to the AR, to both the NTD and the COOH-terminal domain (Cheng et al., 2002).

Furthermore, once the ligand is bound, an intra- and/or inter-molecular interaction takes place between the NH_2-terminal and COOH-terminal domain of the AR, the so-called NH_2-/COOH-terminal domain interaction (Doesburg et

al., 1997; Langley et al., 1995). This interaction, herein abbreviated as NC-TDI, occurs through the FXXLF motif in the NTD (He et al., 2000; Steketee et al., 2002), and also the AF2 AD core plays a role (Berrevoets et al., 1998; Langley et al., 1998; Doesburg et al., 1997; He et al., 1999). Recently, experimental evidence was provided for the preference of the AR binding to FXXLF motifs, by structural predictions based on peptide interactions and on the crystal structure of the LBD containing a bound FXXLF motif containing peptide (He et al., 2004; Dubbink et al., 2004).

The influence on AR target gene expression will change dramatically, if the AR is not functioning properly due to mutations. Mutations resulting in decreased ligand affinity, co-activator interaction or DNA binding, ultimately lead to a decreased AR transcriptional activation potential (http://androgendb.mcgill.ca). Such mutations have been described for 46,XY individuals with the androgen insensitivity syndrome (AIS), ranging from partial AIS (PAIS) to complete AIS (CAIS). AIS is classed as a 46,XY disorder of sex development (DSD) (Quigley et al., 1995; Hughes et al., 2006).

In the present study, a novel AR mutation, F826L, was found in a 46,XY DSD boy with severe penoscrotal hypospadias, possibly associated with PAIS. Hypospadias can result from dysregulation of androgen synthesis or its actions, but is also found without a known cause (Hughes et al., 2006). Several aspects of AR functions were studied to determine the effect of the F826L mutation, which is located in the LBD. The studies were focused on protein expression level and hormone binding characteristics of the mutant AR in genital skin fibroblasts (GSFs) from this boy. In addition, the mutant AR was transiently expressed in CHO and Hep3B cells, to compare its functional

properties to that of wild-type AR, with respect to sub-cellular distribution, hormone responsivity, transactivation potential, NC-TDI, TIF2 co-activation, and repression by N-CoR.

Materials and Methods

Clinical data

The 46,XY boy with the AR mutation F826L was referred to the clinic for a severe penoscrotal hypospadias at the age of 6 months. A mutation in codon 826 of the *AR* was detected (TTC → TTA) resulting in a substitution of a phenylalanine by a leucine residue. The mutation was also identified in the grandmother and the mother of the boy. Sequence analysis of in total 252 alleles (from 82 normal men and from 85 normal women) revealed no alterations at codon 826, indicating that the C → A mutation is not a common AR polymorphism. The poly Gln, Gly and Pro stretches were 22, 17 and 8 residues in length respectively and within the normal range. There were no reports on other affected family members. Furthermore, based on a human chorionic gonadotrophin (hCG) stimulation test at the age of 6 months, well-known causes of 46,XY DSD such as Leydig cell hypoplasia, 17β-hydroxysteroid dehydrogenase (HSD) type 3 deficiency, and 5α-reductase type 2 deficiency were excluded (Boehmer et al., 2001). The penoscrotal hypospadias was successfully corrected in 2 stages at the age of 1.5 - 2 years.

Site-directed mutagenesis and plasmids

All the AR amino acid numbers used in the present study are based on the National Center for Biotechnology Information accession number AAA51729, which refers to the AR of 919 amino acid residues (Lubahn et al., 1988). The TTC → TTA mutation at codon 826 was introduced into the AR cDNA in the pSG5AR construct using QuikChange Site-Directed Mutagenesis (Stratagene,

La Jolla, CA, USA). The mutated AR fragment was excised by EcoR1 digestion and exchanged with the corresponding wild-type fragment in pSG5AR. Ligation was performed with Rapid DNA Ligation Kit (Roche Diagnostics, Basel, Switzerland). This resulted in pAR-F826L. Preparation of the GST-AR.LBD construct was described previously (Steketee et al., 2002). The TTC → TTA mutation was introduced into this construct using the same approach.

The NH_2-terminal domain AR construct pSVAR(TAD1-494) and the COOH-terminal domain AR construct pSVAR-104 (herein indicated as AR-C; aa 537-919) that were used for the NH_2-/COOH-terminal domain interaction and for the TIF2 activation studies were described previously (Doesburg et al., 1997; Jenster et al., 1995). The pSVAR-104 construct encodes the DBD-hinge-LBD regions of the AR. The mutation was introduced into the pSVAR-104 construct via the EcoR1 restriction fragment of pAR-F826L. This resulted in a construct encoding AR-C-F826L.

Generation of pGFP-AR constructs, coding for NH_2-terminally tagged GFP-AR fusion proteins of which the expression is driven by a CMV promoter, has been described previously (Farla et al., 2004). GFP-AR F826L was constructed by replacing an EcoRI-PvuI cDNA fragment encoding the LBD of pGFP-AR by the same fragment of pAR-F826L. All constructs were sequenced to check for errors.

The N-CoR expression vector was constructed as described previously (Berrevoets et al., 2004).

Scatchard plot analysis

A genital skin biopsy was obtained from the boy for the genital skin fibroblasts (GSFs) culture. The GSFs were cultured in minimal essential medium containing 1 % (v/v) nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 10 % (v/v) fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin (BioWhittaker, Verviers, Belgium). For ligand binding characteristics, a whole-cell binding assay was performed as previously described (Bruggenwirth et al., 1996). Briefly, GSFs were cultured to confluency, washed once with PBS, and subsequently incubated overnight in medium without serum. Next, the cells were incubated for 1 h at 37 °C with increasing concentrations of the radiolabeled synthetic androgen ^{3}H-R1881 (NEN Life Science Products, Boston, MA, USA) in the absence or presence of a 200-fold excess of nonradioactive R1881. Cells were then placed on ice, washed 4 times with ice-cold PBS, and subsequently lysed in 0.5 M NaOH. ^{3}H activity in the lysate was measured using a liquid scintillation counter. Scatchard analysis was carried out to determine the equilibrium dissociation constant (K_d) using the Kell software package (Radlig, Biosoft, Ferguson, MO, USA). Protein measurement was performed with the RCDC protein assay according to the instructions from the manufacturer (Bio-Rad, Hercules, CA, USA).

Luciferase assay

For transcription activation studies, CHO cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA), supplemented with 5 % (v/v) fetal calf serum (FCS; Hyclone, Logan, UT, USA) that was dextran-coated charcoal-

stripped, 100 IU/ml penicillin, and 100 µg/ml streptomycin (BioWhittaker, Verviers, Belgium). For all transcription activation studies, CHO cells were plated in 24-well plates (Nalge Nunc International, Naperville, IL, USA) at a density of 2 x 10^4 cells per well. After 24 h, cells were transfected using FuGENE6 reagent (Roche Diagnostics, Basel, Switzerland), according to the instructions of the manufacturer, at a DNA:FuGENE ratio of 1:2. The DNA mixture was composed of 50 ng/well of mouse mammary tumour virus (MMTV)-luciferase (LUC) reporter plasmid, 3 ng/well of SV40-Renilla-LUC, increasing concentrations of either wild-type pSG5AR or pAR-F826L (0.1–3 ng/well), and carrier plasmid pTZ19 to adjust to a total amount of 250 ng DNA per well. Five hours after transfection, 1 nM R1881 or vehicle (0.1 % (v/v) ethanol) was added to the cells, or in the case of dose-response curves a range of 0.1 pM to 1 µM R1881 was added. Testosterone or 5α-dihydrotestosterone were also added in a concentration range of 0.1 pM to 1 µM as indicated in the figures (Steraloids Inc., Wilton, NH, USA). After overnight incubation, cells were lysed in 50 µl lysis LUC buffer [25 mM Tris-phosphate (pH 7.8), 15 % (v/v) glycerol, 1 % (v/v) Triton X-100, and 1 mM dithiothreitol], and 25 µl lysate was used to measure LUC activity. At 10 min after addition of 25 µl Dual-Glo the MMTV-LUC activity was measured. The luciferase reaction was stopped with 25 µl “Stop and Glo” and 10 min thereafter the Renilla-LUC activity was measured (Promega, Madison, WI, USA). The data shown are the mean of at least 2 (T) or 3 (R1881 and DHT) independent experiments (mean ± S.E.M.). For this assay also ARE_2-TATA-LUC was used. This luciferase reporter construct contains 2 AREs in front of the E1b TATA sequence as a promoter (Jenster et al., 1997). To perform both

LUC assays and immunoblots from the same lysates, 12.5 times the usual amount of CHO cells and DNA constructs were used. Thus 250,000 CHO cells were used for every condition of which 20,000 were used for the LUC assay and the remaining cells for the immunoblot.

Western blot analysis

For AR Western blot analysis, GSFs containing either the wild-type AR or the F826L AR were cultured in the presence of unstripped FCS for 7 days, as described above. When grown to confluency, medium was replaced by medium containing 10 % (v/v) dextran-coated charcoal-stripped FCS in the presence of increasing concentrations of R1881 or vehicle (0.1 % (v/v) ethanol) for 24 h, and GSFs were washed with PBS, collected in ice-cold PBS, and centrifuged for 5 min at 800 x g. The cell pellet was resuspended in 100 µl ice-cold RIPA buffer [40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 % (v/v) glycerol, 10 nM sodium phosphate, 10 mM sodium molybdate, 50 mM NaF, 0.5 mM sodium orthovanadate, 10 mM dithiothreitol, 1 % (v/v) Triton X-100, 0.08 % (w/v) SDS, and 0.5 % (w/v) desoxycholate] containing Complete protease inhibitors (Roche Diagnostics) and centrifuged for 10 min at 400,000 x g. GSF cell lysates were loaded onto a 7 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Western immunoblotting was performed using monoclonal antibody F39.4.1 and proteins were visualized by Western Lightning chemiluminescence detection (Perkin-Elmer, Boston, MA, USA).

Western immunoblotting of CHO cells was performed by using lysates from the corresponding LUC assay, by immunoprecipitation with the

monoclonal AR antibody F39.4.1 followed by SDS-PAGE, and subsequent detection with polyclonal antibody SP197 targeting the NH_2-terminal domain of the human AR (Kuiper et al., 1993).

NH_2-/COOH-terminal domain interaction (NC-TDI) assay and TIF2 activation assay

The functional NC-TDI assay and TIF2 activation assay were performed in essentially the same way as the transactivation assay described above, except for the used constructs. For the NC-TDI assay, 100 ng/well of the NH_2-terminal domain AR construct AR-N (pSVAR(TAD1-494)) (Doesburg et al., 1997) was used in combination with increasing concentrations of the COOH-terminal domain AR construct AR-C (pSVAR-104) (Jenster et al., 1995) or AR-C-F826L (0.3–30 ng/well).

For TIF2 co-activation assay, 100 ng/well of TIF2 expression vector was added in combination with increasing concentrations of constructs encoding AR-C or AR-C-F826L (0.3–30 ng/well).

N-CoR repression and N-CoR-TIF2 competition assay

The functional N-CoR repression and N-CoR-TIF2 competition assays were performed by essentially the same procedure as the transactivation assay described above. For the N-CoR repression assay, different amounts of N-CoR expression constructs were transfected together with 3 ng of either wild-type pSG5AR or pAR-F826L expression constructs. For the N-CoR-TIF2 competition assay, 30 ng/ well of TIF2 and/or N-CoR was used. pSG5 vector

was added to obtain equal molar fractions of plasmid in each well for both assays.

GST pull-down assay

In vitro interaction assays (pull-down assays) were performed as described previously (Steketee et al., 2002). In short, CHO cells were transfected with pSVAR(TAD1-494) and either GST-AR.LBD-wt or GST-AR.LBD-F826L. After overnight incubation in medium supplemented with 100 nM R1881 or vehicle, cells were lysed and rotated for 5 h at 4 °C with glutathione-agarose beads. Next, beads were washed, subsequently boiled in Laemmli sample buffer, and subjected to SDS-PAGE. After Western blotting, visualization of pSVAR(TAD1-494) was performed with the AR antibody SP197, and of GST-AR.LBD with the AR antibody SP066 (Jenster et al., 1995). The expression of each protein was semi-quantified by Quantity One® (Bio-Rad, Hercules, CA, USA). The relative NC-TDI was determined as the ratio between the blotted protein amounts of the NH_2-terminal domain and the corresponding COOH-terminal domain. The ratio of the wild-type AR-LBD expression vector was set at 1 and the ratios for 3 AR F826L isolates were determined relative to that of the wild-type AR-LBD.

Confocal microscopy of GFP proteins

Hep3B cells were cultured in αMEM (Cambrex, East Rutherford, NJ) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 5 % (v/v) FCS (PAN Biotech GmbH, Aidenbach, Germany). Two days before confocal microscopy, cells were seeded on glass coverslips

in six-well plates. One day prior to confocal microscopy, medium was substituted by medium supplemented with 5 % dextran charcoal-treated FBS. Four hours after medium change cells were transfected with 1 µg/well GFP-AR expression constructs in FuGENE6 (Roche) transfection medium. Four hours after transfection, the medium was replaced by medium containing 5 % dextran-coated charcoal-stripped FCS with or without 1 nM R1881. Live-cell imaging was performed using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany) using the 488 nm laser line of a 30 mW Ar laser with tube current set at 6.1 A. Cells were kept at 37 °C. Images were obtained using a 40 X /1.3 NA oil immersion lens using 505-530 nm emission filters.

Protein structure

The three-dimensional (3D) crystal structure of the AR ligand binding pocket complexed with R1881 was obtained from the Protein Data Bank (PDB; accession no. 1XOW) deposited in the data bank by He et al. (2004). The diagram showing the LBD and selected residues that were subject to mutation was created using the ViewerLite 5.0 program from Accelrys. The distance between amino acid residues and the amino acid interaction surface were predicted by the ligand-protein contacts (LPC) software (Sobolev et al., 1999).

Results

Ligand binding and expression of AR F826L in genital skin fibroblasts

To determine whether the expression of the F826L AR mutant in GSFs was affected by the mutation, an immunoblot was performed after incubation of GSFs with different concentrations of R1881 or vehicle for 24 h. The protein expression level of AR F826L in GSFs appeared to be similar at every R1881 concentration (Figure 1A). Scatchard plot analysis revealed a K_d value of 0.073 nM for AR F826L versus a K_d value of 0.058 nM for AR wild-type (normal range of 0.03 to 0.13 nM)(Figure 1B). The number of binding sites in the GSFs of AR F826L was 57.2 fmol/mg and for AR wild-type 61.2 fmol/mg (normal range 39 - 169 fmol/mg)(Bruggenwirth et al., 1997). In conclusion, the F826L mutation in the AR did not influence the expression level and ligand binding properties of the AR.

Transcriptional activation and expression of AR mutant F826L

The ligand-induced transactivation activity of the AR mutant F826L appeared to be similar to that of the wild-type AR with the MMTV-LUC reporter construct (Figures 2A and 2B). Similar data were found with the minimal promoter construct ARE_2-TATA-LUC (data not shown). To exclude ligand specificity of the mutant the ARE_2-TATA-LUC reporter was also tested in the presence of 5α-dihydrotestosterone (DHT). Also under these conditions the transactivation of the AR mutant F826L was found to be identical to that of the wild-type AR (data not shown).

To study whether the mutation affected AR stability, an immunoblot was performed with cell lysates used in the luciferase assay presented in Figure 2A. The expression level of AR mutant F826L protein in CHO cells was only slightly higher, compared to the AR wild-type expression level (Figure 2C). It can be concluded that the mutation F826L did not influence the transactivation function and stability of the AR protein.

Sub-cellular distribution of AR mutant F826L

The sub-cellular localisation of the AR mutant F826L, was determined in the presence and absence of hormone by confocal microscopy after transfection of GFP-AR and GFP-AR F826L expressing constructs into Hep3B cells. Both GFP-AR and GFP-AR F826L were predominantly located in the cytoplasm in the absence of hormone (Figures 3A and 3B). In the presence of 1 nM R1881 both GFP-AR and GFP-AR F826L were translocated in a similar way to the nucleus and displayed a typical punctuate nuclear distribution pattern (Figures 3C and 3D). This typical speckled pattern indicates an active transcription of endogenous genes (Farla et al., 2005; van Royen et al., 2007). It can be concluded that the F826L mutation did not influence the sub-cellular distribution of the AR mutant F826L.

Functional NH_2-/COOH-terminal domain interaction (NC-TDI) of AR mutant F826L

NH_2-/COOH-terminal domain interaction (NC-TDI) is an important parameter of AR function. The F826L mutant was tested for this interaction. CHO cells were co-transfected with AR-NH_2-terminal domain, MMTV-LUC,

and an increasing amount of either wild-type AR-COOH-terminal domain AR-C or AR-C-F826L expression vectors. In the presence of 1 nM R1881, AR-C-F826L displayed an almost two-fold increase in NC-TDI as compared with wild-type AR (Figure 4A; $p<0.01$). Immunoblots of cell lysates used in the LUC-assay of Figure 4A showed that the expression of AR-C-F826L was not different from that of the wild-type AR-C (Figure 4B).

The effect of different R1881 concentrations on the NC-TDI was compared with that of testosterone (T) and of 5α-dihydrotestosterone (DHT) with the same assay and with an ARE_2-TATA-LUC reporter. At 0.1 nM or higher concentrations of R1881, T as well as DHT, the transactivation by AR-C-F826L was increased, as compared with the transactivation by wild-type AR (Figures 5A, B and C). 0.1 nM T induced a smaller increase as compared with DHT and R1881 for both the wild-type and mutant AR, indicating and confirming the relative lower affinity of the mutant and wild-type receptors for T. It can be concluded that all three hormones (R1881, T and DHT) display an at least two-fold increase in the NC-TDI assay for the mutant AR-C-F826L as compared with the wild-type AR-C. A similar increase for the mutant AR was also observed with MMTV-LUC as reporter in the presence of DHT or R1881 (data not shown).

GST pull-down assay of AR F826L

To confirm the increased NC-TDI of AR mutant F826L, a GST-pull-down assay was performed with lysates from CHO cells transfected with the AR NH_2-terminal domain expression vector and one of the GST-AR.LBD constructs. The experiment was performed in triplicate with 3 different GST-

AR.LBD-F826L cDNA isolates. After GST pull-down, SDS-PAGE and immunoblotting, the relative NC-TDI in the presence of 1 nM R1881 was determined as the ratio between the blotted protein amounts of the NH_2-terminal domain and the corresponding COOH-terminal domain. The ratio for the wild-type AR-LBD expression vector was arbitrarily set at 1 and the ratios for the 3 AR F826L isolates (clones 1, 2 and 3) were determined relative to that of the wild-type AR-LBD. The assay revealed that at least twice the amount of the NH_2-terminal domain was pulled down by the 3 GST-AR.LBD-F826L isolates as compared with the wild-type AR-LBD (Figure 6 and Table 1).

TIF2 co-activation of AR mutants F826L

Interaction of the co-activator TIF2 with the COOH-terminal domain of the AR can provide additional information on AR function (Berrevoets et al., 1998). To examine activation of the AR mutant F826L by TIF2, an expression vector encoding this co-activator was co-transfected with AR-C-F826L. The co-activation of AR-C-F826L was significantly higher ($p<0.01$), up to 2-fold compared to the result obtained for the wild-type AR-C (Figure 7A).

The same assay was performed with increasing concentrations of R1881, testosterone and 5α-dihydrotestosterone. From 0.1 nM onwards the TIF2 co-activation of AR-C-F826L by R1881 and DHT was higher as compared with that of the wild-type AR-C (Figures 7B and 7D). TIF2 co-activation in the presence of T was higher from 1 nM onwards for the mutant AR (figure 7C). This indicates and confirms again the relative lower affinity of T for both the mutant and wild-type AR. It can be concluded that in the presence of either

R1881, T or DHT co-activation by TIF2 of the AR-C-F826L was at least two-fold higher than of the wild-type AR-C. A similar increase for the mutant AR was also observed with MMTV-LUC as reporter in the presence of R1881 (data not shown).

Repression of mutant F826L by N-CoR, in absence and/or presence of TIF2

Since the ligand binding affinity and the transactivation activity of AR F826L were comparable to that of the wild-type AR, and the NC-TDI and the co-activation by TIF2 of mutant F826L were increased, the question arose whether the severe penoscrotal hypospadias of the boy could be explained by preferential binding of co-repressors by the F826L mutant. To answer this question, CHO cells were transfected with either the wild-type AR or AR mutant F826L with increasing concentrations of N-CoR expression vector (0-30 ng/well), in the presence of 1 nM R1881. However, in the presence of N-CoR, the decrease in transactivation of wild-type AR and AR F826L was not different (Figure 8A).

The present experiments on the effects of TIF2 co-activation and N-CoR repression involved relatively high expression levels of these proteins. Differences between the AR mutant F826L and AR wild-type, regarding differential affinities for TIF2 or N-CoR, may go unnoticed in such an analysis. Therefore, we investigated competition between N-CoR and TIF2, in CHO cells transfected with AR constructs, and with different combinations of expression constructs for N-CoR or TIF2. The transactivation observed in the presence of N-CoR alone decreased with approximately 35 %, for both wild-

type AR and AR F826L (Figure 8B). If both N-CoR and TIF2 expression vectors were added, activity could be relieved to 80 to 85 % for both AR wild-type and AR F826L (Figure 8B). In the presence of TIF2 alone, the activity increased for both AR wild-type and AR F826L to approximately 160 % (Figure 8B).

Discussion

The investigated F826L mutation in the AR was found in a 3-year old boy with severe penoscrotal hypospadias. The grandmother and mother of the boy both were carriers of this genetic alteration. Sequence analysis of 252 alleles (from 82 men and 85 women) and information from the AR gene mutations database (http://androgendb.mcgill.ca/) revealed that alterations at codon 826 do not occur in the general population. Therefore it is highly unlikely that the mutation at codon 826 of the *AR* gene in this individual reflects a common polymorphism in the *AR* gene.

Remarkably, the activity of AR mutant F826L was identical to the wild-type AR with respect to ligand binding, number of ligand binding sites in GSFs, transactivation and repression. The repression by N-CoR of both the wt AR and AR mutant F826L could be relieved by TIF2, although not to 100%. This indicates that repression by N-CoR is stronger than co-activation by TIF2 under these conditions, which confirms a previous study for only the wild-type AR (Berrevoets et al., 2004).

It was thought that AR F826L was more abundantly present in the nucleus as compared to wild-type AR, resulting in a similar outcome of transcription activity measurements, even if AR F826L would be less active itself. However, this can likely be ruled out, since the sub-cellular distribution was found to be similar for AR F826L and wild-type AR. Furthermore, after addition of R1881, GFP-AR and GFP-AR F826L displayed a typical punctuate nuclear distribution pattern, as previously shown by Farla et al. (2004, 2005) for the wild-type receptor. This typical speckled pattern overlaps with sites of active transcription (van Royen et al., 2007).

Interestingly, this study showed that the NC-TDI of the AR mutant F826L was increased compared to the wild-type AR and this increase was shown with 2 different assays, 2 different promoters and 3 different ligands. This increase in activity could not be explained by a higher protein stability of the AR mutant. Furthermore, the TIF2 co-activation of AR mutant F826L was also enhanced compared to the wild-type AR. These experiments also confirmed the lesser potency of T versus DHT, because higher T concentrations were needed to achieve the same transcriptional activity as with DHT (Askew et al., 2007). In addition, the NC-TDI might be very sensitive to subtle changes in the AR ligand binding domain. Also for other mutations, information on NC-TDI and TIF2 co-activation can be of much value to detect a possible difference between wild-type and mutant AR. In contrast to our study, several studies showed that AR mutations found in subjects with AIS and prostate cancer, can result in an altered NH_2-/COOH-terminal domain interaction and co-factor interaction. Five mutations resulting in AIS, L712F, F725L, I737T, Q733H and I898T respectively, are located in the hydrophobic region of AF2, which all result in a defective NC-TDI and in a defective interaction with TIF2 (Quigley et al., 2004; He et al., 2006). In addition, the AR mutants F725L and I737T have a defective interaction with SRC1 (Quigley et al., 2004). AR mutations L907F and R885H, which both can result in an defective NC-TDI, are also found in close proximity of the AF2 (Jaaskelainen et al., 2006). Another mutation Q902K, also located close to AF2, displays a defective NC-TDI and an increased R1881 equilibrium dissociation constant (Umar et al., 2005). An affected NC-TDI and TIF2 interaction is also found for two other mutations close to AF2, G743V (PAIS) and V889M (CAIS) (He et al., 2006;

Thompson et al., 2001). In close proximity of G743, three mutations are reported in AIS subjects, R871G, S814N and V866M, which were found to have a defective NC-TDI (Ghali et al., 2003). The M745I mutated residue from a CAIS subject is part of the ligand binding pocket, and causes a defective NC-TDI and a defective interaction with ARA70 (Bonagura et al., 2007). Remarkably, this mutation does not affect the interaction of AR with TIF2 and SRC1, whereas the R1881 equilibrium dissociation constant is increased 5 times compared to wild-type AR (Bonagura et al., 2007). Mutations D695N, Y763C, E772A, R774H, R774C and Q798E from AIS subjects are all located on the surface of the LBD at a relatively large distance from AF2, but surprisingly all mutants display a defective NC-TDI (Ghali et al., 2003; Jaaskelainen et al., 2006). Three of these residues (D695, Y763 and R774) together with residues R752 and F754 have been suggested to form a new region for protein-protein interactions, although this is not supported by experimental data (Jaaskelainen et al., 2006). Another mutation, R855H, found in an AIS individual is located also at a large distance from the AF2 region, within helices 10/11 which contain residues of the ligand binding pocket (Matias et al., 2000). This mutant displays a decreased NC-TDI, but also an increased androgen equilibrium dissociation constant (Elhaji et al., 2004). For prostate cancer, several mutations (V715M, R726L, H874Y) have been reported displaying either an increased NC-TDI or an increased p160 co-activator activation or both (Thompson et al., 2001; Duff and McEwan, 2005; He et al., 2006; Brooke et al., 2008). The mutation V715M resulted in a small increase of NC-TDI, but in a normal p160 co-activator activation, while for the mutant R726L the reverse was found (Thompson et al., 2001).

However, in another study the mutant V715M has been reported to display an increased NC-TDI as well as an increased p160 co-activator interaction (He et al., 2006). The mutant H874Y has been studied extensively and this mutant displayed an increased p160 co-activator interaction (Brooke et al., 2008; Duff and McEwan, 2005; He et al., 2006). Contradictory results were reported with respect to the NC-TDI. Either wild-type activity (Duff and McEwan, 2005) or a significant increase was found with the H874Y mutant (He et al., 2006).

Summarising it can be concluded that mutations in or near the AF2 region predominantly negatively influence the protein interaction with AF2. However, for certain mutations in prostate cancer a positive influence on protein interactions with AF2 is reported. Furthermore, mutations in the LBD at a far more distance from AF2 and not influencing ligand binding affinity, seem to influence the protein interaction with AF2, as well.

What might be causing the increased NC-TDI and the increased activation by TIF2 observed for the mutant AR F826L? Although the 3D model for the AR-LBD predicts that residue F826 is neither part of the ligand binding pocket nor the co-activator binding groove, this mutation might still influence the co-activator binding groove indirectly. The ligand-protein contacts (LPC) software predicts that F826 can have hydrophobic interactions with residues N727 and L728 (Sobolev et al., 1999). The distance to N727 is 3.5 Å and the distance to L728 is 3.8 Å. However, the contact surface area between F826 and N727 is about 4 times larger than between F826 and L728, which would indicate that F826-N727 might give the strongest interaction. Residues N727 and L728 are both located in the loop region between helices 3 and 4 (Figure 9) (Wurtz et al., 1996). Changes in the interaction between F826 and either N727 or L728

due to the F826L mutation could have consequences for positions of residues in the LBD structure including those of the co-activator groove. Since residues in helix 3 (K717 and K720) and in the loop region between helices 3 and 4 (R726) belong to the co-activator binding groove. A subtle rearrangement in the F826-N727 or F826-L728 interaction might have a considerable impact on the co-factor interaction.

Most importantly, it has been suggested that residue N727 plays a role in AR transcriptional activation, based on the presence of an AR mutant N727K found in an individual with PAIS (Yong et al., 1994). Other data indicated that N727 could influence NC-TDI and TIF2 co-activation (Lim et al., 2000). Similar to the present findings for the AR F826L mutant, Lim et al. (2000) found that, the N727K AR mutant has unaltered transcriptional activation and ligand binding activities, but increased NC-TDI and TIF2 co-activation activities, compared to wild-type AR. However, this was observed using the synthetic androgen mesterolone, (1-α-methyl DHT). Remarkably, when testosterone or DHT was used, the transcriptional activation, NC-TDI and TIF2 co-activation of the AR N727K mutant were found to be decreased, compared to the wild-type AR (Lim et al., 2000). It appears that the effect of the N727K mutant depends on the type of ligand used. But in the present study, this was not the case. The synthetic androgen R1881 generated similar results as testosterone and 5α-dihydrotestosterone.

Recently, Estebanez-Perpina et al. (2007) showed that residue F826 is part of an allosteric regulatory site termed binding function (BF)-3 in the AR LBD. Mutagenesis of residues that form BF-3 modulated AR function (Estebanez-Perpina et al., 2007). The AR F826A mutant showed normal, wild-

type level of AR transcriptional activity (Estebanez-Perpina et al., 2007). However, another mutant, F826R displayed a small decrease (15 %) in transcriptional activity, probably caused by the change in hydrophobicity (Estebanez-Perpina et al., 2007). The F826L mutation changes the hydrophobic phenylalanine to an even more hydrophobic leucine (hydropathy index changes from 2.8 to 3.8)(Kyte and Doolittle, 1982). The mutant residue F826A has become less hydrophobic (hydropathy index of 1.8) and the mutant residue F826R became even lesser hydrophobic (hydropathy index of -0.8). The hydrophilic arginine has probably caused the 15 % decrease in transcriptional activation activity. Such a change in hydrophobicity does not seem to be notable in a functional assay with the full-length mutant AR. For the AR mutant N727K, the change to a very hydrophilic residue decreased the transcriptional activity (Lim et al., 2000).

The increase in hydrophobicity of the F826-N727 interaction appeared to be associated with an enhanced transcriptional activity whereas a decreased hydrophobicity is associated with a reduced transcriptional activity. Consequently, the observed increased NC-TDI and TIF2 co-activation in the present experiments might be caused by the increased hydrophobicity of the leucine residue of AR mutant F826L.

Another question that remains, concerns the factor(s) causing the severe penoscrotal hypospadias phenotype in the boy with the AR mutation F826L. Several reports described AR mutations in helix 9 in the region surrounding F826 that resulted in AIS. For example, Q824L was identified in a boy with a partial androgen insensitivity syndrome. This mutation resulted in a decreased AR activity, depending on the type of ligand used. In the presence of DHT, 62

% of wild-type activity was observed, whereas with the synthetic androgen mibolerone, no difference in activity was found (Giwercman et al., 2000). Another mutation close to the position of F826, F827V, found in an individual classified as having CAIS, was reported to result in just 20 % less ligand binding affinity in genital skin fibroblasts (Chavez et al., 2001a). The AR mutant L830V was also found in a CAIS individual (Chavez et al., 2001b). These facts suggest that mutations in the region of F826 result in AIS (ranging from partial to complete). It has been shown that different cell types have a cell-specific expression of co-factors (Folkers et al., 1998; Shang and Brown, 2002). The severe penoscrotal hypospadias observed in the boy carrying the F826L AR mutation might be the result of a combination of a different repertoire of tissue specific co-factors and a subtle alteration of the co-activator binding groove. Consequently this can result in less recruitment of important key co-activators, other than TIF2, in genital skin fibroblasts. Alternatively, an altered LBD conformation may enhance a preferential recruitment of co-repressor(s), present in specific androgen target cells.

In conclusion, the mutant F826L displayed an unexpected increased NH_2-/COOH-terminal domain interaction and TIF2 co-activation. These findings cannot directly explain the observed severe penoscrotal hypospadias, but offer indirect indications for an altered AR functioning.

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Table 1

	N/C ratio[a]	relative to wt[b]
Wild-type AR	0,1	1
AR F826L clone 1	0,6	4,7
AR F826L clone 2	0,3	2,2
AR F826L clone 3	0,3	2,7

[a]The relative NC-TDI from Figure 6 was determined as the ratio between the blotted protein amounts of the NH_2-terminal domain (N) and the corresponding COOH-terminal domain (C) in the pull-down fraction.

[b]The ratio of the wild-type (wt) AR was arbitrarily set at 1 and the ratios of the 3 AR F826L isolates (clones 1, 2 and 3) were determined relative to the wt AR.

Figure 1

Expression and ligand binding properties of AR F826L in genital skin fibroblasts (GSFs)

(**A**) AR protein expression levels were analysed in GSFs after incubation for 24 h in the presence of R1881 (1, 10, and 100 nM) or vehicle alone (0.1 % ethanol). An amount of 12 μg lysate was subjected to SDS-PAGE and immunoblotting with AR monoclonal antibody F39.4.1.

(**B**) Ligand binding properties of AR F826L were determined by Scatchard analysis, after incubation of GSFs with 1 - 0.5 - 0.25 - 0.1 - 0.05 - 0.025 nM ^{3}H-R1881 for 1 h. The dissociation constant (K_d) was determined with the KELL program (Biosoft® KELL).

Figure 2

Transcriptional activation of AR mutant F826L

(**A**) CHO cells were transfected with increasing amounts (0.1, 0.2, 0.3, 0.4, 1 and 3 ng/well) of DNA plasmids expressing either wild-type (wt) AR or AR mutant F826L, both in combination with 50 ng of the reporter construct MMTV-LUC and 3 ng/well of Renilla luciferase. The cells were harvested 16 h after treatment with 1 nM R1881 or vehicle and luciferase activity was measured. Represented are the means ± S.E.M. for 3 separate experiments. The MMTV-LUC activity was normalized with the Renilla LUC activity and the activity of 1 ng wt AR/well was set at 100 %. Fold induction is shown at the top of each bar and represents the mean ratio of activity determined after

incubation in the presence or absence of R1881. Statistical significance was calculated using the Student's t-test ($p<0.01$).

(**B**) Dose-response curves of the wt AR and AR F826L constructs (1 ng/well) with increasing concentrations of R1881. LUC activity of the wt AR at 1 nM R1881 was set at 100 %.

(**C**) Part of the cell lysates used in the LUC assay were used for immunoblotting. Cell lysates of CHO cells transfected with different amounts of DNA and incubated with 1 nM R1881 were immunoprecipitated with monoclonal AR antibody F39.4.1. The precipitate was thereafter subjected to SDS-PAGE and immunoblotting with polyclonal AR antibody SP197.

Figure 3

Sub-cellular distribution of AR mutant F826L

Confocal laser-scanning microscope images of Hep3B cells transfected with 1 µg of GFP-AR or GFP-AR F826L in the absence of ligand (3A and 3B) or in the presence of 1 nM R1881 (3C and 3D). The bars represent 10 µm.

Figure 4

NH_2-/COOH-terminal domain interaction of AR mutant F826L

(**A**) CHO cells were transfected with 50 ng of reporter construct MMTV-LUC, 3 ng/well of Renilla luciferase, and different amounts (0, 1, 3, 10, and 30 ng) of DNA plasmid for either wt COOH-terminal domain construct AR-C or the mutant construct AR-C-F826L, both together with 100 ng of AR NH_2-terminal domain construct pSVAR(TAD1-494) (AR-N). The cells were harvested 16 h after incubation with 1 nM R1881 or vehicle, and luciferase

activity was measured. Results represent data of 3 experiments (means ± S.E.M.). Fold induction is shown at the top of each bar and represents the mean ratio of activity determined after incubation in the presence or absence of R1881. The MMTV-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 ng DNA/well was set at 100 %. Statistical significance was calculated between wt and mutant AR using the Student's t-test ($p<0.01$) and is indicated with asterisks (*).

(**B**) Part of the cell lysates used in the LUC assay was used for immunoblotting. Lysates of CHO cells transfected with AR COOH-terminal domain constructs (30 ng/well) and incubated with 10 nM R1881 were immunoprecipitated with monoclonal AR antibody F52.24.4 in the presence of 0.3 M NaCl. Subsequently, the immunoprecipitate was subjected to a 10 % SDS-PAGE gel electrophoresis and immunoblotted with polyclonal AR antibody SP066 against the COOH-terminal domain.

Figure 5

Dose response of the NH_2-/COOH-terminal domain interaction of AR mutant F826L

(**A**) NC-TDI of mutant F826L and wt AR COOH-terminal domain (10 ng/well) with AR NH_2-terminal domain (100 ng/well) in the presence of increasing concentrations of R1881. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM was set at 100 %. Results are based on at least 3 experiments (mean ± S.E.M.).

(**B**) NC-TDI of mutant F826L and wt AR COOH-terminal domain (10 ng/well) with AR NH_2-terminal domain (100 ng/well) in the presence of increasing concentrations of testosterone. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM was set at 100 %. Results represent data of 2 experiments (mean ± S.E.M.).

(**C**) NC-TDI of mutant F826L and wt AR COOH-terminal domain (10 ng/well) with AR NH_2-terminal domain (100 ng/well) in the presence of increasing concentrations of 5α-dihydrotestosterone. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM was set at 100 %. Results rare based on at least 3 experiments (mean ± S.E.M.).

Figure 6

GST pull-down assay of F826L

Proteins were produced in CHO cells by transfection of AR-N (1 µg) and co-transfected with either the wt GST-AR.LBD construct or one of the 3 isolates of the GST-AR.LBD-F826L constructs (3 µg). After overnight incubation in the absence or presence of 100 nM R1881 the cells were lysed. Subsequently, the pull-down assay and SDS-PAGE was performed, followed by immunodetection with AR antibody SP197 against the NH_2-terminal domain and AR antibody SP066 against the COOH-terminal domain.

Figure 7

TIF2 co-activation of AR mutant F826L

(**A**) CHO cells were transfected with 50 ng of reporter construct MMTV-LUC, 3 ng/well of Renilla luciferase, and different amounts of DNA plasmids from either wt COOH-terminal construct AR-C or mutant AR-C-F826L, both together with 100 ng of a construct encoding TIF2. The cells were harvested 16 h after incubation with either vehicle or 1 nM R1881, and luciferase activity was measured. Results represent data of 3 experiments (means ± S.E.M.). Fold induction is shown at the top of each bar and represents the mean ratio of activity determined after incubation in the presence or absence of R1881. The MMTV-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 ng DNA/well was set at 100 %. Statistical significance was calculated between wt and mutant AR using the Student's t-test ($p<0.01$) and is indicated with asterisks (*).

(**B**) Dose-response curve of the 2 AR COOH-terminal constructs (10 ng/well) in the presence of TIF2 expression vectors (100 ng/well) and increasing concentrations of R1881. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM R1881 was set at 100 %. Results represent data of at least 3 experiments (means ± S.E.M.).

(**C**) Dose-response curve of the 2 AR COOH-terminal constructs (10 ng/well) in the presence of TIF2 expression vectors (100 ng/well) and increasing concentrations of T. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM T was set at 100 %. Results represent data of at least 3 experiments (means ± S.E.M.).

(**D**) Dose-response curve of the 2 AR COOH-terminal constructs (10 ng/well) in the presence of TIF2 expression vectors (100 ng/well) and increasing concentrations of DHT. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM DHT was set at 100 %. Results represent data of at least 3 experiments (means ± S.E.M.).

Figure 8

N-CoR repression of AR mutant F826L

(**A**) CHO cells were transfected with 50 ng of reporter construct MMTV-LUC, 3 ng/well of Renilla luciferase, and different amounts of N-CoR DNA plasmids together with 3 ng of either wild-type AR or pAR-F826L constructs. In the control situation, equal molar fractions of empty vector (pSG5) were transfected. The cells were harvested 16 h after treatment with vehicle alone or 1 nM R1881, and luciferase was measured. Results represent data of 3 experiments (means ± S.E.M.). The MMTV-LUC activity was normalized with the Renilla LUC activity. The minus N-CoR situation for each AR construct was set to 100 % from which the relative repression by N-CoR was calculated.

(**B**) Competition between TIF2 (30 ng/well) and N-CoR (30 ng/well). Except for the different combinations of transfected constructs, the assay was performed as described above (A). In the control situation, equal molar fractions of empty vector (pSG5) were transfected and the outcome was set at 100 %. Results represent data of 3 experiments (means ± S.E.M.). The MMTV-LUC activity was normalized with the Renilla LUC activity.

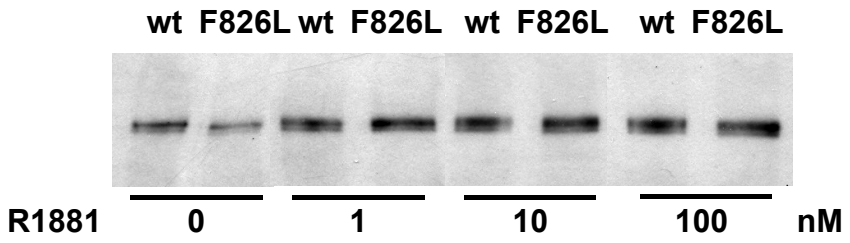
Figure 9

Position of AR F826 in the AR LBD with respect to the co-activator binding groove

Structural model of the AR LBD with liganded R1881 in the ligand binding pocket. Residue F826 might have hydrophobic interactions with residues N727 and L728; the distances are 3.5 and 3.7 Å, respectively. N727 and L728 are both in the loop region between helices 3 and 4. R726 in the loop region and K717 and K720 in helix 3 belong to the co-activator binding groove. The AR FXXLF peptide is shown to indicate the co-activator binding groove. K720 and E897 represent the charge clamp residues essential for binding of FXXLF and LXXLL motifs.

Figure 1

(A)



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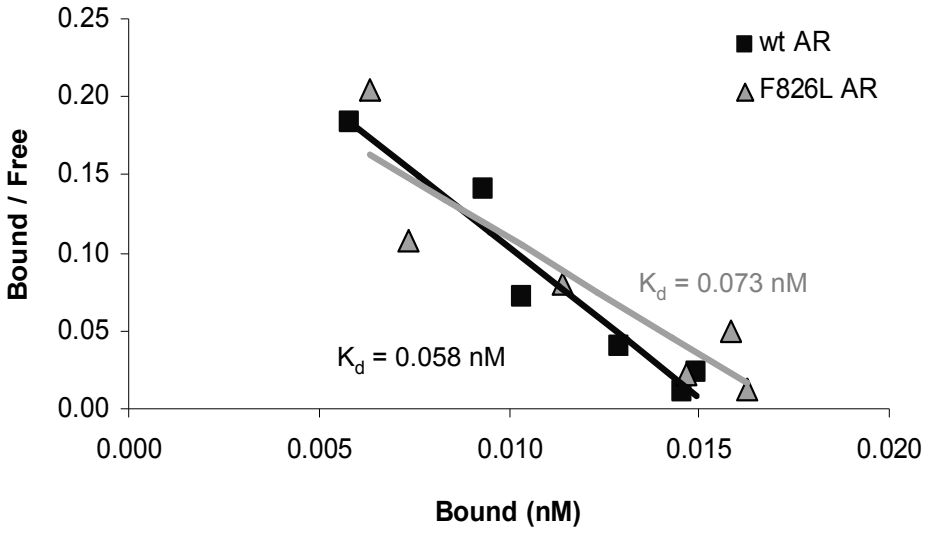
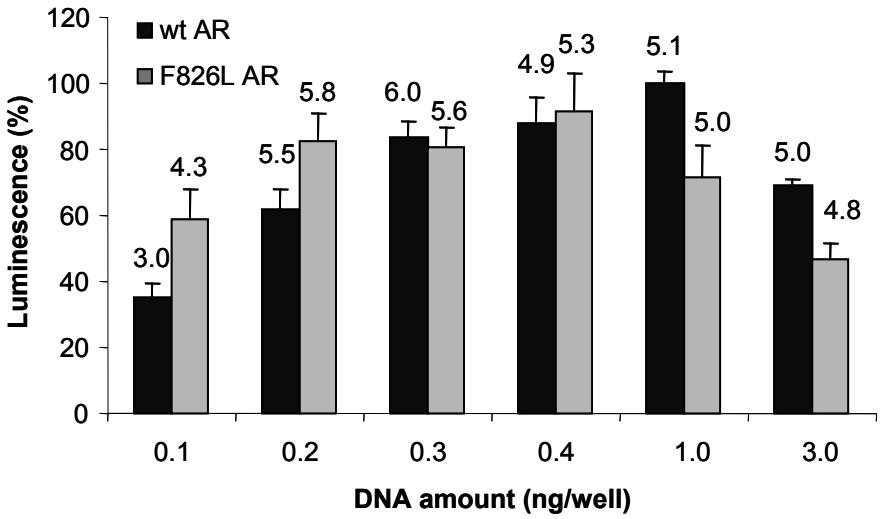


Figure 2

(A)



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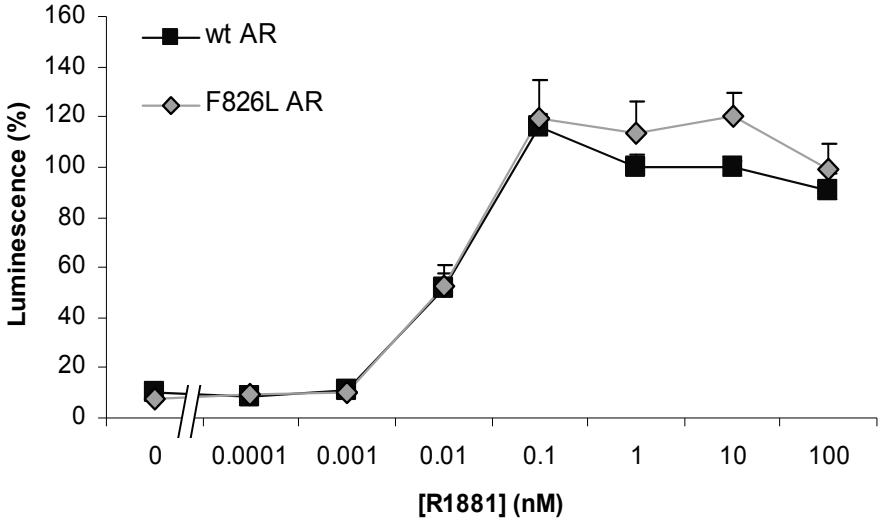

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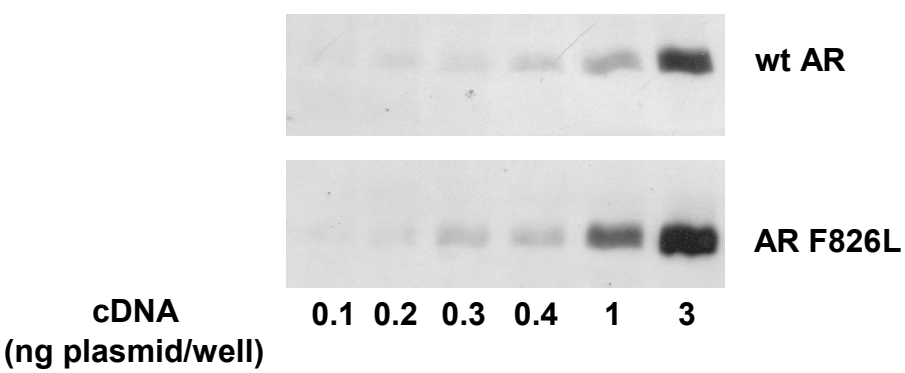


Figure 3

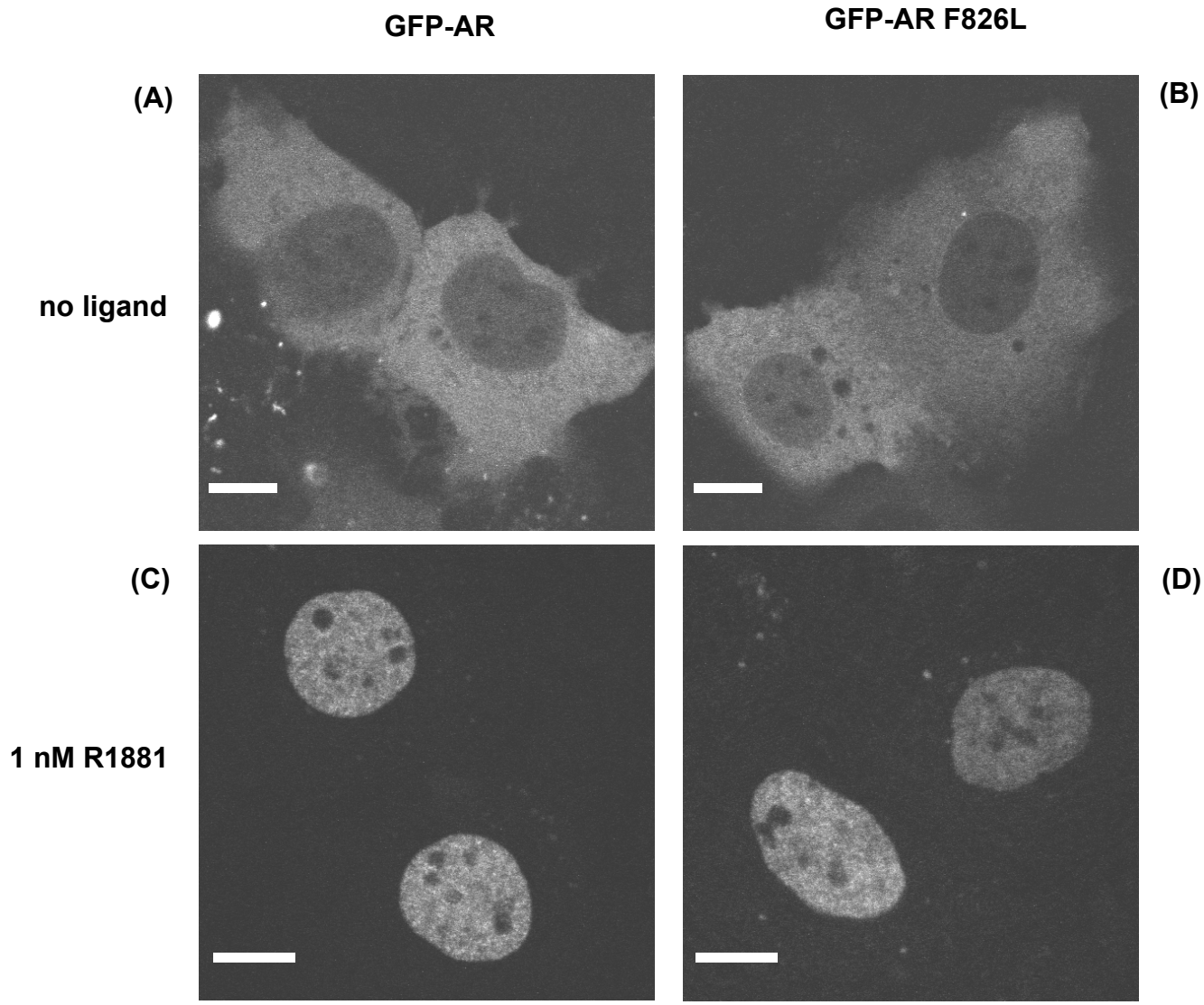
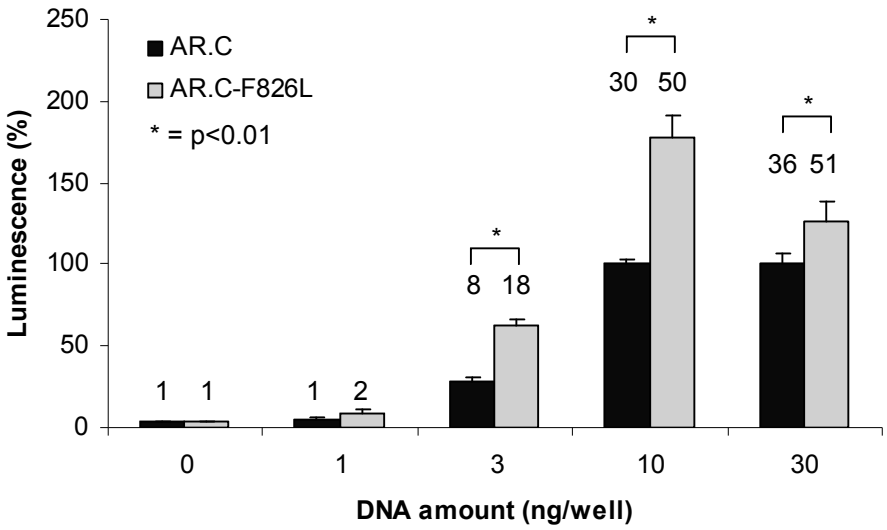


Figure 4

(A)



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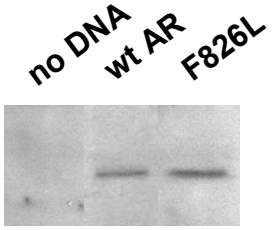


Figure 5

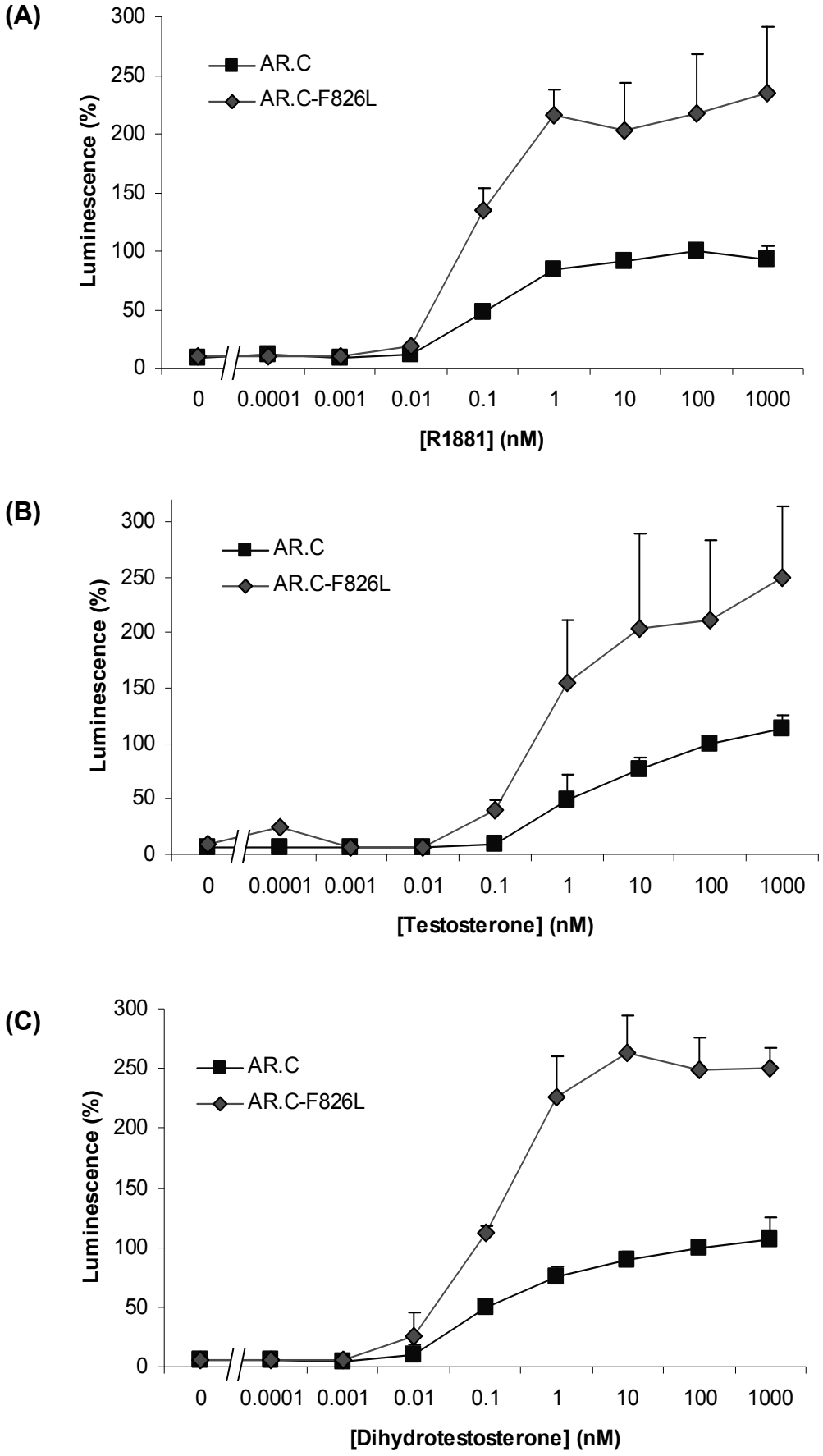


Figure 6

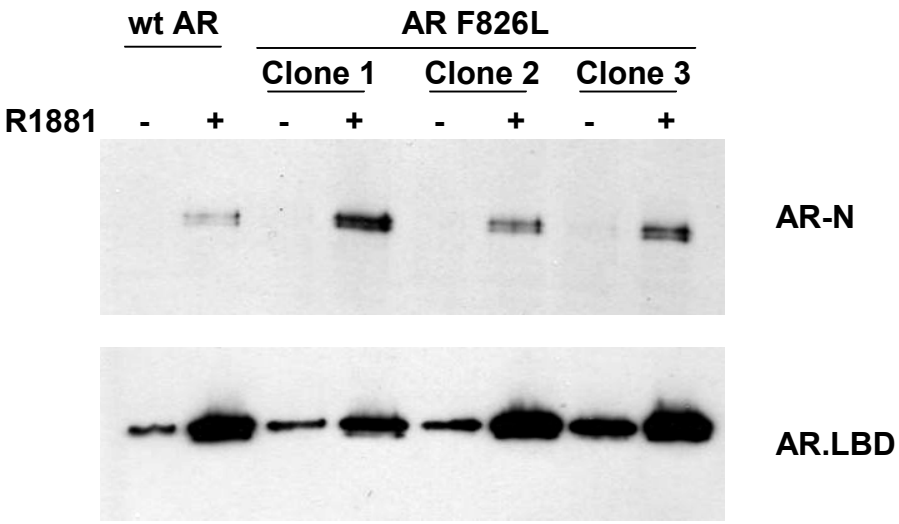


Figure 7

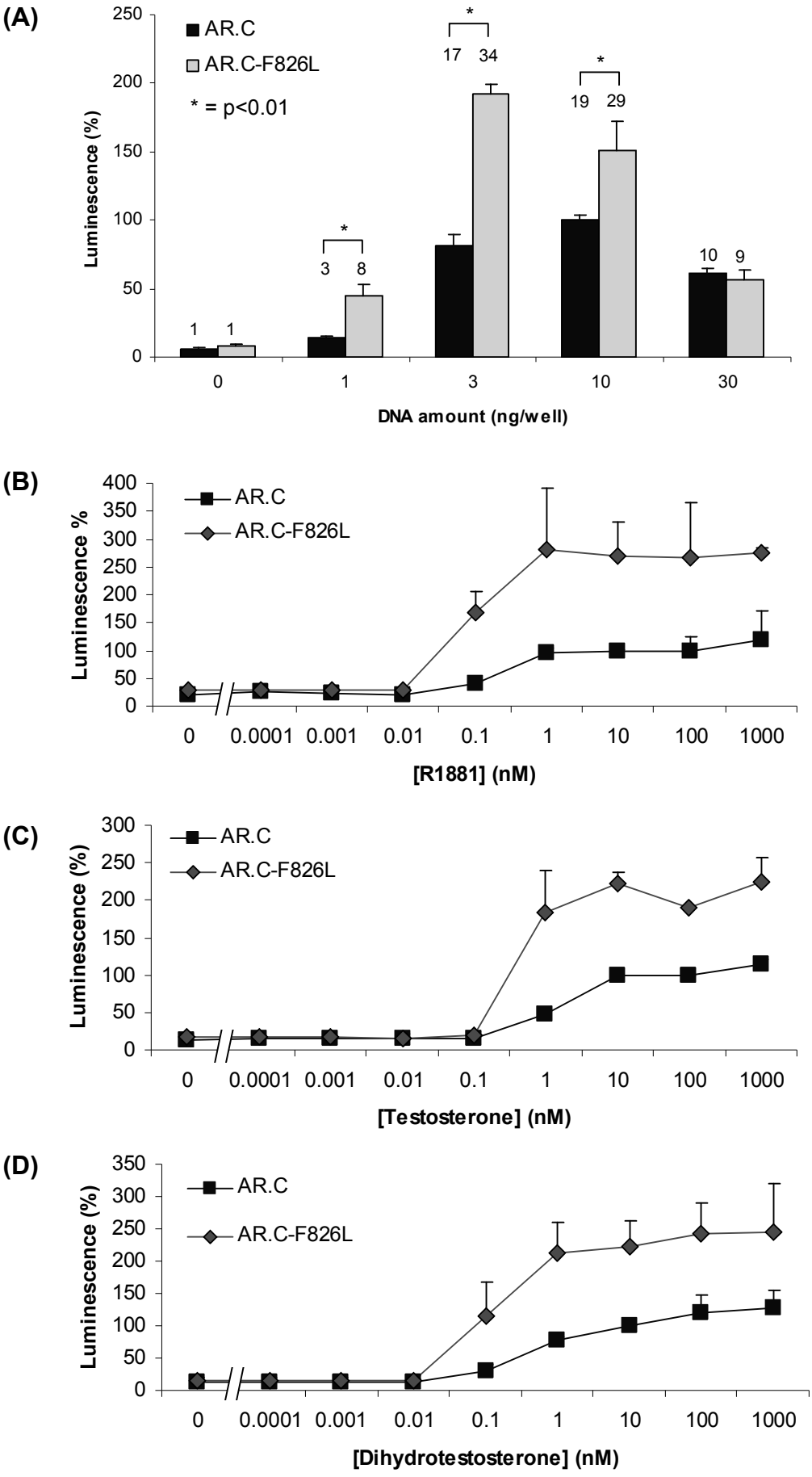
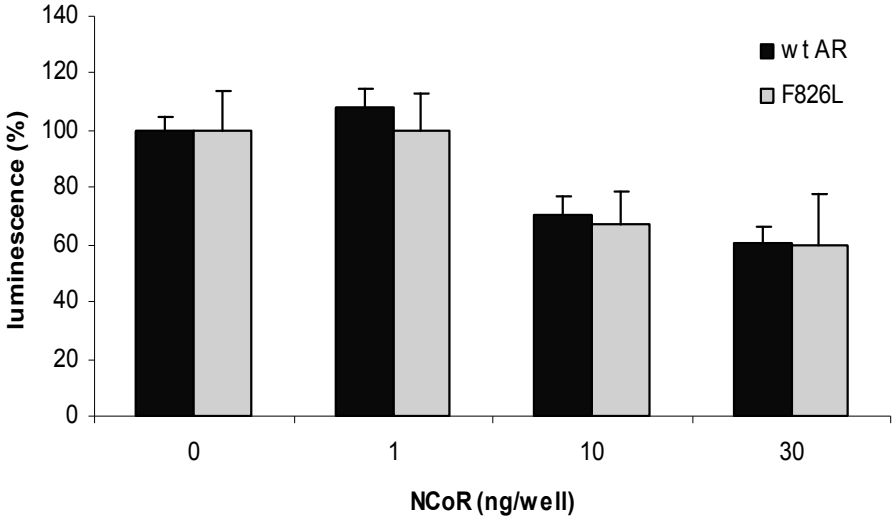


Figure 8

(A)



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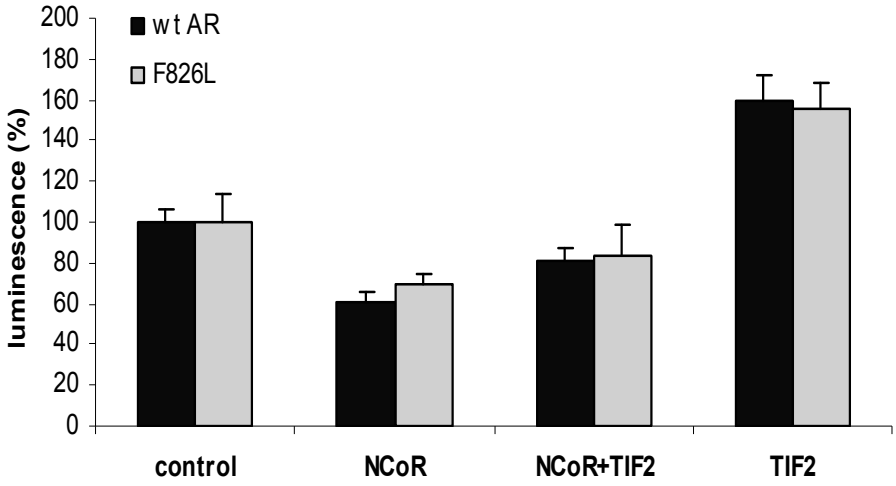

Figure 9