

AD \_\_\_\_\_

Award Number: W81XWH-09-1-0630

TITLE: N-Cadherin in Prostate Cancer: Downstream Pathways and Their Translational Application for Castrate-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Robert Reiter

CONTRACTING ORGANIZATION: University of California, Los Angeles  
Los Angeles, CA 90095

REPORT DATE: September 2010

TYPE OF REPORT: Other

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Unclassified  
Distribution Statement: Unclassified

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.



## Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	16
Reportable Outcomes.....	17
Conclusion.....	18
References.....	19
Appendices.....	20

## INTRODUCTION

Radical prostatectomy and radiation therapy are effective treatments for primary prostate cancer, although some patients will experience disease recurrence. Standard treatment for recurrent prostate cancer is androgen deprivation therapy (ADT), which is initially very effective in causing the tumors to shrink and disappear. Over time (months to years), however, tumors become resistant to this therapy and re-emerge in an androgen-independent form (i.e., capable of growing in the apparent absence of androgens) called castration-resistant prostate cancer (CRPC). These tumors will metastasize to other tissues, particularly bone. Molecular events underlying the transition from androgen-dependent to castration-resistant prostate cancer are poorly understood. To identify pathways that contribute to castration resistance, we compared gene expression patterns of androgen-dependent prostate cancer cells transplanted in either castrated or matched androgen-dependent xenograft mice. With subsequent transplantation into castrated mice, we found that the tumors became increasingly metastatic and the expression of one gene, a cell surface protein called N-cadherin, rose consistently with each subsequent passage. Moreover, high levels of N-cadherin expression were found in tumor tissues of patients treated with ADT and patients with CRPC, compared with very low levels of N-cadherin in patients with androgen-dependent prostate cancer. These data suggest that increased N-cadherin expression is involved in the development of castration resistance in prostate cancer patients.

## **Aim 1: N-cadherin signaling: roles of the NF- $\kappa$ B, PI3K/Akt, and AR pathways**

### **1.1 Determine the components of the NF- $\kappa$ B pathway that are activated by N-cadherin**

Collaborating PI, Dr. Rettig presented these data on establishing the specific IKK isoforms and NF- $\kappa$ B family members activated by N-cadherin in his first annual report, which is summarized here. We have previously shown by luciferase reporter assay that NF- $\kappa$ B activity is upregulated in N-cadherin overexpressing cells. We further reported the constitutive NF- $\kappa$ B activity in these overexpressing cell lines compared to N-cadherin negative LNCaP cells, as evident by heightened NF- $\kappa$ B binding in electrophoretic mobility shift assay (EMSA) and IKK activation. We identified IKK $\beta$ , p65 and p50 as being activated in N-cadherin cells. We also demonstrated that NF- $\kappa$ B inhibition by either a pharmacologic IKK $\beta$  inhibitor or a molecular I $\kappa$ B super repressor attenuated invasion in N-cadherin expressing cells. In addition, NF- $\kappa$ B blockade induced re-expression of E-cadherin protein, a marker of epithelial phenotype as opposed to the invasive mesenchymal phenotype of N-cadherin overexpressing cells.

### **1.2 Assess the interactions between N-Cadherin, NF- $\kappa$ B, AKT and AR signaling pathways**

Both sub-tasks were completed in the first year and re-summarized here for the final report. N-cadherin overexpressing cells showed constitutively upregulated NF- $\kappa$ B activity compared to N-cadherin negative LNCaP cells, as evident by heightened NF- $\kappa$ B binding in electrophoretic mobility shift assay (EMSA) and IKK activation specifically IKK $\beta$ , p65 and p50. Inhibiting NF- $\kappa$ B activity either pharmacologically or molecularly attenuated invasion in N-cadherin expressing cells, confirming the link between N-cadherin and NF- $\kappa$ B activation. N-cadherin overexpressing cells exhibited increased Akt activity, while inhibiting PI3K/Akt signaling pathway caused specific inhibition of NF- $\kappa$ B activity. N-cadherin expressing cells that have undergone EMT showed low or undetectable AR expression, suggestive of an inverse relationship between Ncadherin and AR. This was confirmed in our *in vivo* model, LAPC9 xenografts serially passaged in castrated mice, which over time showed increased N-cadherin with decreased AR. However, subpopulations of tumor cells co-expressing both N-cadherin and AR existed in earlier passage, suggesting that dual targeting approach is viable.

### **1.3 Determine the interactions between blockade of N-Cadherin and downstream pathways in combination on *in vitro* biology**

We reported on *in vitro* studies using monoclonal antibody 2A9 against N-cadherin extracellular domain 4 in combination with PI3K inhibitor LY294002 (LY). Treatment reduced invasion, with LY being more effective, and combination of both 2A9 and LY showed additive effect by suppressing cell invasion further. TGF- $\beta$  induced invasion was markedly reduced by either 2A9 or LY, and combination treatment again showed additive effect of suppressing invasion. Cell growth inhibition using either N-cadherin antibody 2A9, or 1H7 which targets extracellular domains 1-3, was only evident after 7 days of treatment. However, LY treatment alone significantly inhibited cell growth after 2 days compared to antibody treatment, and combination of LY with either antibody did not further inhibit growth. TGF- $\beta$  treatment did not affect cell growth while EGF treatment markedly induced growth by 30%. 1H7 was more effective than 2A9 in reducing this EGF-induced growth increase, but LY treatment was much more effective than either antibody. Combination of LY and either antibody did not show additive inhibition of EGF-induced growth.

As previously reported, we showed that IKK $\beta$  inhibitor reduced invasion of N-cadherin overexpressing cells and that the inhibitor of both IKK $\alpha$  and IKK $\beta$  isoform inhibits cell proliferation as effective as LY, but there was no additive inhibition when treatment was combined with 2A9 or 1H7 antibody. Treatment with IKK $\alpha$  specific inhibitor did not reduce cell growth, confirming that the reduced cell growth resulted from inhibition of NF- $\kappa$ B signaling is through IKK $\beta$ . The data suggested that N-cadherin specific antibody attenuates cancer cell invasion through the Akt signaling pathway and the synergistic effect of inhibiting both N-cadherin and Akt activity is also significant in reducing TGF- $\beta$  induced invasion. In contrast, N-cadherin antibody did not affect cell proliferation at the time point when PI3K inhibitor and IKK inhibitor were evidently inhibiting and there was no further synergy in combination treatment.

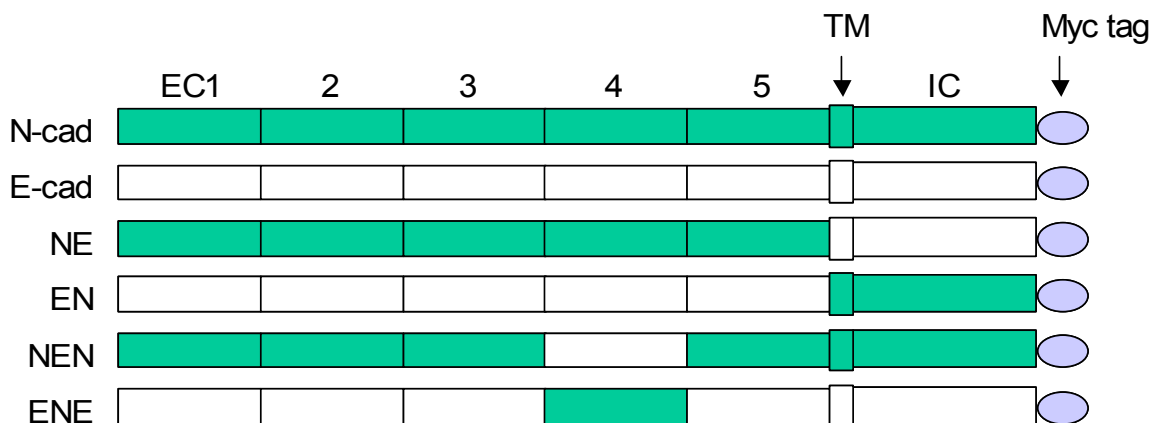
We also assessed the effects of inhibiting both N-cadherin and androgen receptor (AR) using LNCaP<sub>Ncad</sub>, in which full length N-cadherin-myc tag was overexpressed to be used as one control subline in Aim 2. This subline still expresses abundant AR and serves as a good model to study effects of treatment with N-

cadherin antibody and the AR antagonist MDV3100. LNCaP-Ncadmyc (LN-Ncad) subline grew faster than LNCaP control line (LN) in regular medium. MDV3100 inhibited growth in both cell lines dose-dependently, but was significantly more inhibitive in LN than LN-Ncad. Treatment of LN-Ncad cells with 2A9 in the presence of 1 $\mu$ M MDV3100 showed synergistic growth inhibition while each agent alone did not show inhibitive effect. However at higher dose of 10 $\mu$ M, MDV3100 alone was equally effective and there was no additive effect in combination with 2A9.

## **Aim 2: Role of the extracellular and intracellular domains of N-cadherin on EMT, castration resistance and NF- $\kappa$ B activation**

### **2.1 Construction and transfection of chimeras**

As reported before, three chimeric cadherin sublines and two control sublines were generated and used in subsequent experiments. These included LNCaP cell lines (LN) stably expressing the chimeric constructs NE-myc, NEN-myc, EN-myc, and the full length Ncad-myc, Ecad-myc.



**Figure 1.** Illustration of N-cadherin and E-cadherin chimeric proteins. EC: extracellular domain, TM: transmembrane domain, IC: intracellular domain. Green: N-cadherin cDNA, white: E-cadherin cDNA. Each recombinant protein has a myc tag at carboxy-terminal for detection.

### **2.2 Assess the contribution of N-cadherin domains on vitro and in vivo invasion, metastasis and androgen independence**

In motility assay, LN-NE was as efficient as LN-Ncad, as expected from previous report [4], suggesting that similarly in this cell line the extracellular domain (ECD) of N-cadherin was responsible for cell motility. The intracellular domain of N-cadherin was able to induce migration (LN-EN versus LN-O) but not as effective as the ECD. The previous report identified ECD4 of N-cadherin as being sufficient to promote motility [4]. In our hands, replacing N-cadherin ECD4 with E-cadherin ECD4 did not cause reduction in motility (LN-NEN versus LN-Ncad). On the other hand, the invasiveness of these sublines was ranked as followed: LN-Ncad > LN-NEN > LN-NE > LN-EN > LN-O. Here intact N-cadherin was most effective in inducing invasion, and replacement of any Ncadherin domain resulted in reduction of invasion. However, the most reduction was observed in LN-EN, suggesting that the N-cadherin ECD is the major inducer of invasion. Of note, LN-NEN caused the least reduction in invasion, suggesting that in this cell system the N-cadherin ECD4 may contribute to invasive behavior but it alone is not sufficient to confer this phenotype. We repeated assays for in vitro growth of the chimeric cadherin sublines in regular and androgen-depleted conditions. All chimeric sublines and LN-Ncad showed increased proliferation over LN-O control in regular medium, ranking as followed: LN-Ncad = LN-NEN > LN-NE = LN-EN > LN-O. Under androgen depleted condition, the chimeric sublines showed increased growth over negative control line, but not as much as LNCaP expressing intact N-cadherin, with the following rank: LN-Ncad > LN-NEN = LN-NE = LN-EN > LN-O. Thus it was unclear which domain of N-cadherin alone was more effective in conferring androgen independent growth. To assess the effects of the chimeric cadherin constructs on castration resistant growth in vivo, these sublines were implanted subcutaneously into castrated mice and tumor growth monitored over time. Any domain

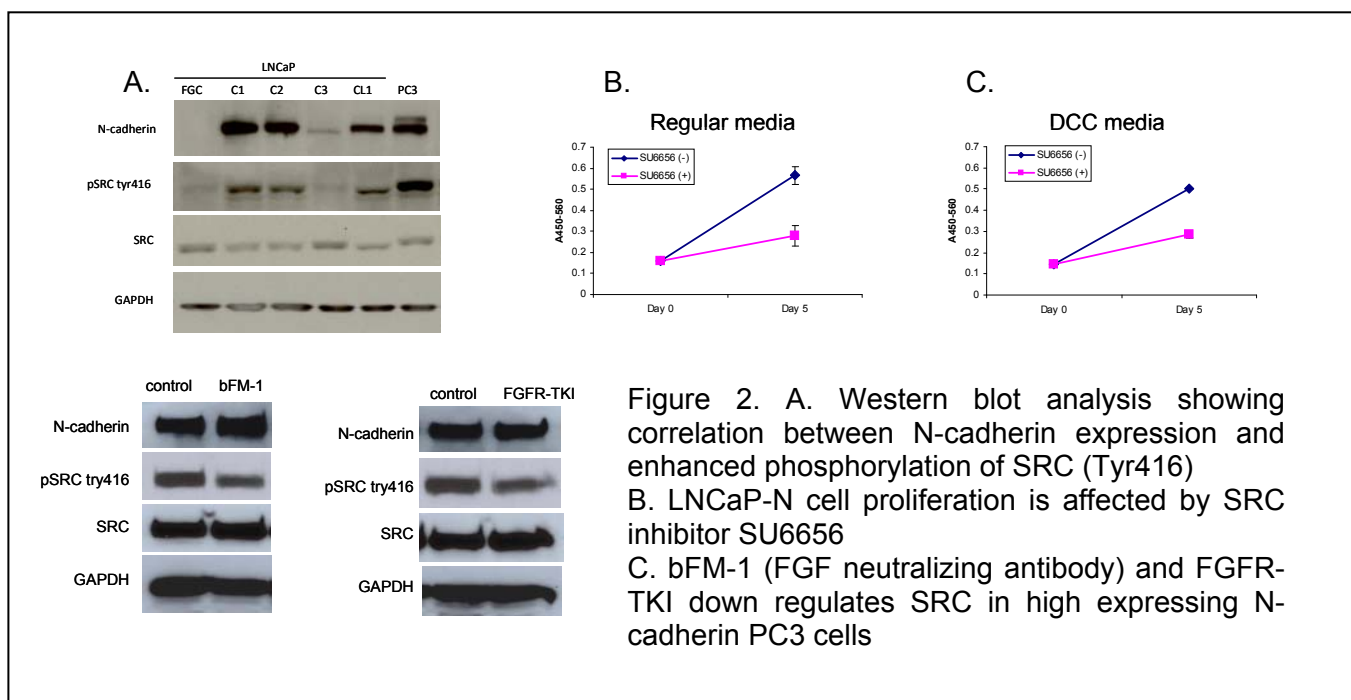
replacement resulted in growth attenuation of 50% or more compared to LN-Ncad, but either ECD or intracellular domain (ICD) of N-cadherin was sufficient to confer castration resistant growth compared to LN-O control. Based on the tumor sizes at the end of the experiment, these sublines could be ranked as followed: LN-Ncad > LN-NEN > LN-NE > LN-EN > LN-O. The results suggested that N-cadherin ECD4 is important but not a requirement for castration resistant growth.

### 2.3 Assess the contribution of N-cadherin domains on gene expression and downstream signal transduction

As stated in the first report, the chimeric cadherin sublines and the intact LN-Ncad line did not undergo EMT like morphologic change. In all sublines, there was no de novo expression of vimentin, repression of E-cadherin, or change in phospho-Akt level in vitro. Vimentin staining was present most abundantly in LN-Ncad tumor, followed by LN-NEN, LN-NE and LN-EN castration resistant tumors. It appeared that vimentin induction was most impaired in LN-EN subline, suggesting that the N-cadherin ECD is required for the signaling events that promotes vimentin expression and potentially EMT. Vimentin staining in LN-NEN tumor was more abundant than LN-EN and LN-NE, suggesting that the N-cadherin ECD4 is not crucial for vimentin induction and potentially EMT. On the other hand E-cadherin and AR were still highly expressed in all tumors, suggesting that N-cadherin could promote castration resistant growth of prostate cancer cells without full transition from epithelial to mesenchymal phenotype.

LN-Ncad, LN-NE, LN-EN and LN-O control cell lines were maintained in androgen-depleted media and stimulated with androgen analogue R1881 to look at effect of N-cadherin and domains of AR activity. Results suggested that in this model N-cadherin overexpression results in androgen insensitivity, since AR expression is induced under androgen-depleted condition and R1881 treatment did not further induce AR level. AR expression is inducible by R1881 in LN-NE and -EN sublines, suggesting that either the ECD or ICD N-cadherin alone is still responsive to R1881 stimulation and therefore may contribute to the tumor growth retardation observed in vivo in castrated mice.

N-cadherin expression activates key pathways implicated in EMT. Some highly expressed genes of interest involved in signaling pathways implicated in EMT are members of the MAPK pathway including FGF-2, FGFR1 (further discussed in Aim 3: Biomarkers) and NFkB pathways (IL-6, IL-8, TLR4 (TLR4 will be further discussed in Aim 3: Combinations)). (Figure 3) Treatment of N-cadherin expressing cells with FGFR and NFkB small molecule inhibitors reduced invasion and in some cases reversed EMT in these cells. We also saw activation of SRC in the N-cadherin positive models consistent with its known role upstream of the MAPK pathway as well as EMT (Figure 2).



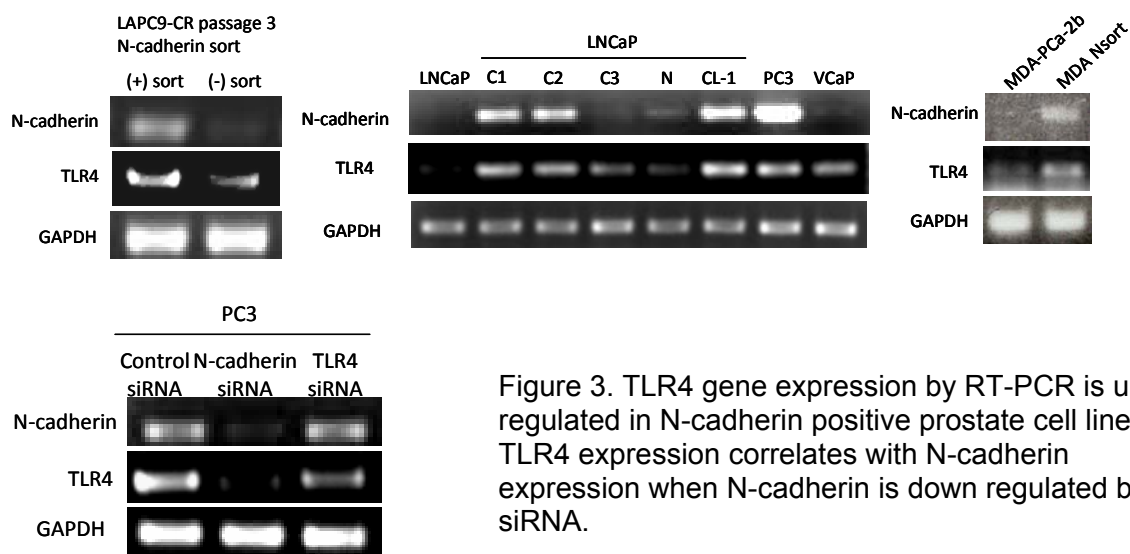


Figure 3. TLR4 gene expression by RT-PCR is up-regulated in N-cadherin positive prostate cell lines. TLR4 expression correlates with N-cadherin expression when N-cadherin is down regulated by siRNA.

### Aim 3: Preclinical studies of N-cadherin antibodies:

Oncologic successes have occurred in the context of multi-agent chemotherapy. Conversely, single agent treatment regimens typically have modest effects. We have tried to build our understanding of the biochemical signaling events that occur as a result of N-cadherin expression to identify “druggable” biochemical targets that can be exploited therapeutically in conjunction with our N-cadherin antibody to achieve additive or synergistic anti-tumor activity.

#### 3.1 Preclinical Models

The goal of this proposal is to clinically translate N-cadherin targeted therapy. In order to assess responses to N-cadherin mono and combination therapy, we used both engineered and endogenous models of N-cadherin expression. For endogenous models, we used PC3 and LAPC9 AD (androgen dependent) and LAPC9 CR (castration resistant). For engineered models, we focused on MDA-N-cadherin retaining low levels of AR and LNCaP-N-cadherin (1 and 3) with LNCaP-N-cadherin 1 expressing high levels of N-cadherin and loss of AR and E-cadherin while LNCaP-N-cadherin-3 expresses lower levels of N-cadherin and retains AR and E-cadherin. We assessed for the ability of the antibodies to inhibit growth, cause regression, and block metastasis of established tumors in castrated mice. (Figure 4)

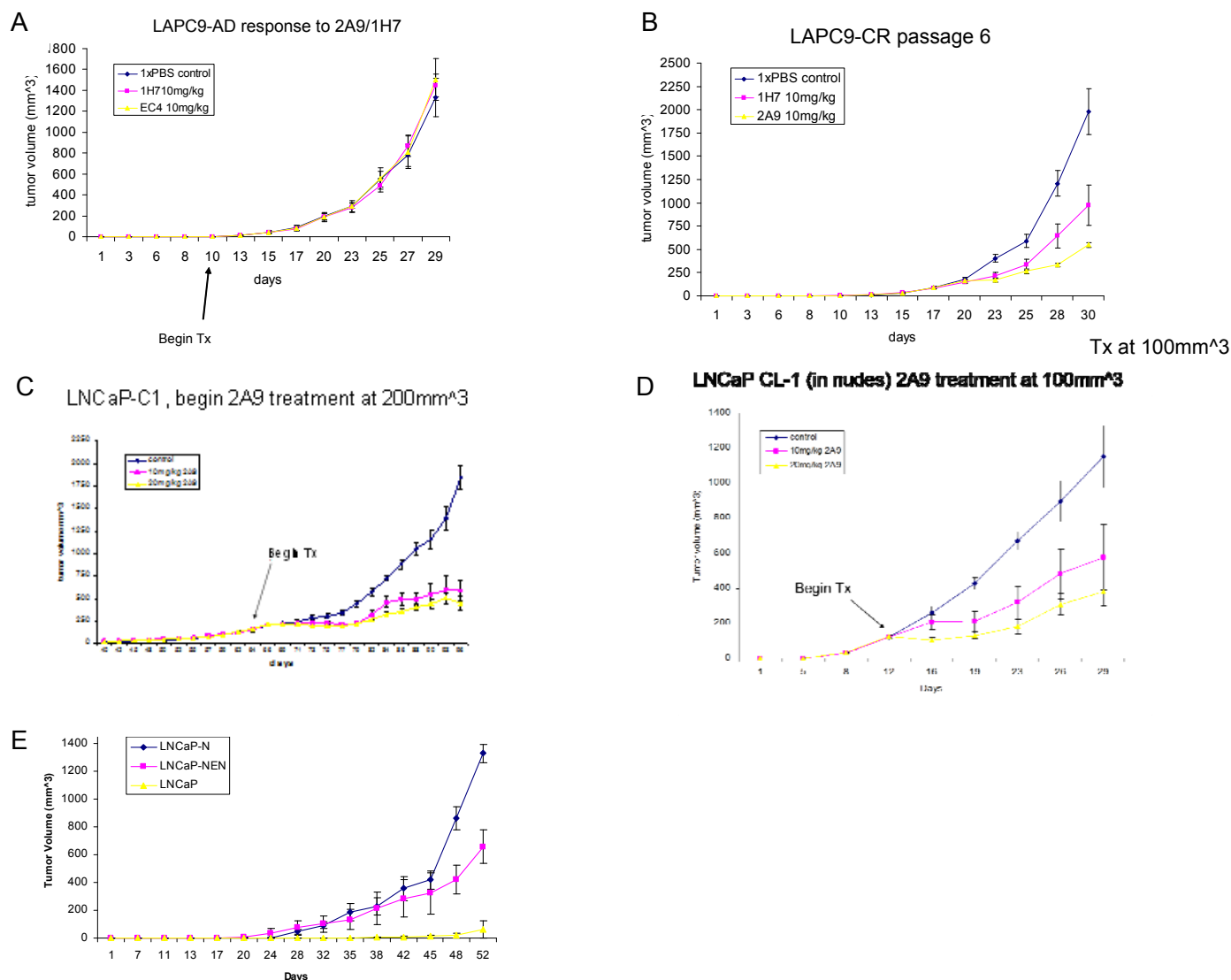


Figure 4. A, B, D: Endogenous in vivo models for N-cadherin expression treated with N-cadherin antibodies. C: Engineered in vivo models of N-cadherin expression treated with N-cadherin antibody. E: Pilot test on in vivo models of chimeric sublines in castrated mice (no ab treatment). All models had inhibition of tumor growth with treatment. LAPC9-AD expresses little to no N-cadherin and therefore should not respond to treatment.

### 3.2 N-cadherin antibody studies

We have generated two N-cadherin antibodies that have significant anti-tumor activity. 1H7 targets ECD1-3 and 2A9 (aka EC4) targets ECD 4. We evaluated the activity of single and combined therapy for anti-tumor effect. (Figure 5)

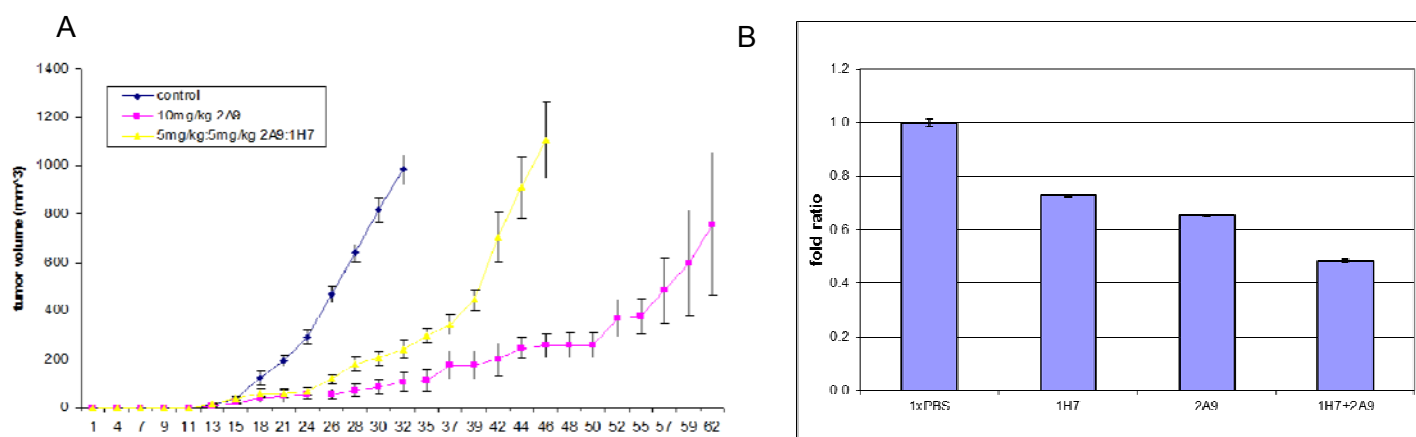


Figure 5. A. Using a model tested in 3.1, we evaluated combined therapy on PC3 tumors in vivo. Unfortunately, due to technical errors, we will need to repeat this model along with testing a second model. B. In vitro invasion assay on PC3 cells with single and combined therapy. The combination therapy appears to slightly increase inhibition of invasion.

### 3.3 Combinations

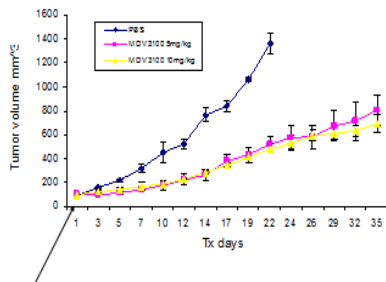
Ncadherin expression induces activation of at least three critical signaling pathways that have been identified as drivers of castration resistant prostate cancer growth: (1) the nuclear factor kappa B (NF-κB) pathway (2) the PI3K/AKT pathway, and (3) the FGFR-MAPK pathway. In addition, persistent AR activation as a driving biochemical force behind castration resistance, are the basis for the selection of the agents used in our proposed drug combination studies. In addition, we have selected these combinations because drugs with effects on these pathways are already used in men with advanced prostate cancer, or are currently in clinical trials.

N-cadherin expression is associated with marked changes in gene expression related to androgen regulation. The metastatic lesions of CRPC patients have been shown to exhibit heterogeneity of AR staining [4, 5] with some lacking AR expression (N-cadherin positive) while others express in the nucleus (N-cadherin negative) all within the same tissue slide. MDV3100, a novel AR antagonist and is approximately five times higher binding affinity to AR in comparison to bicalutamide was tested in combination with our N-cadherin antibody to try and target this mixed population. (Figure 6)

As discussed in Aim 2, TLR4 is a highly expressed gene of interest possibly involved in the NFκB signaling pathway implicated in EMT. N-cadherin expression in prostate cell lines appears to correlate with resistance to Docetaxel. TLR4 upregulation correlates with N-cadherin expression. siRNA knockdown of N-cadherin and 2A9 antibody treatment reverses this resistance. Combination therapy using 2A9 and Docetaxel show some additive effect. siRNA knockdown of TLR4 and HTA125 (TLR4 neutralizing antibody) treatment in N-cadherin positive cell lines show sensitivity to Docetaxel.

Preliminary data suggests that N-cadherin affects chemoresistance by the TLR4 pathway in a MyD88 dependent manner. It suggests that Docetaxel activates NFκB by MyD88-dependent TLR4 pathway in N-cadherin positive cells. NFκB transcriptional target IL-8 secretion (increased secretion reported to cause resistance to cisplatin and paclitaxel in ovarian cancer) is affected by Docetaxel treatment and is reversed by blocking TLR4 activity by siRNA or HTA125. (Figures 7 and 8)

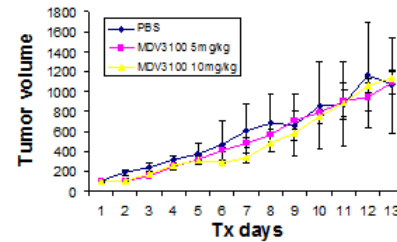
## LAPC9-CR passage 3



Tx a

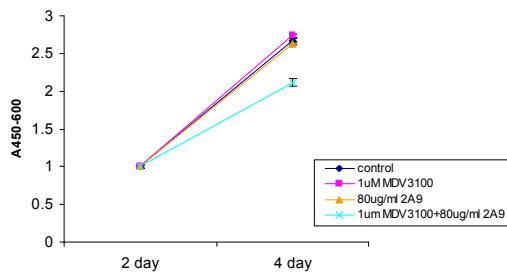
Figure 6. Treatment of LAPC-9CR passage 3 expressing both N-cadherin and AR with MDV3100 shows partial sensitivity.

## LAPC9-CR passage 5

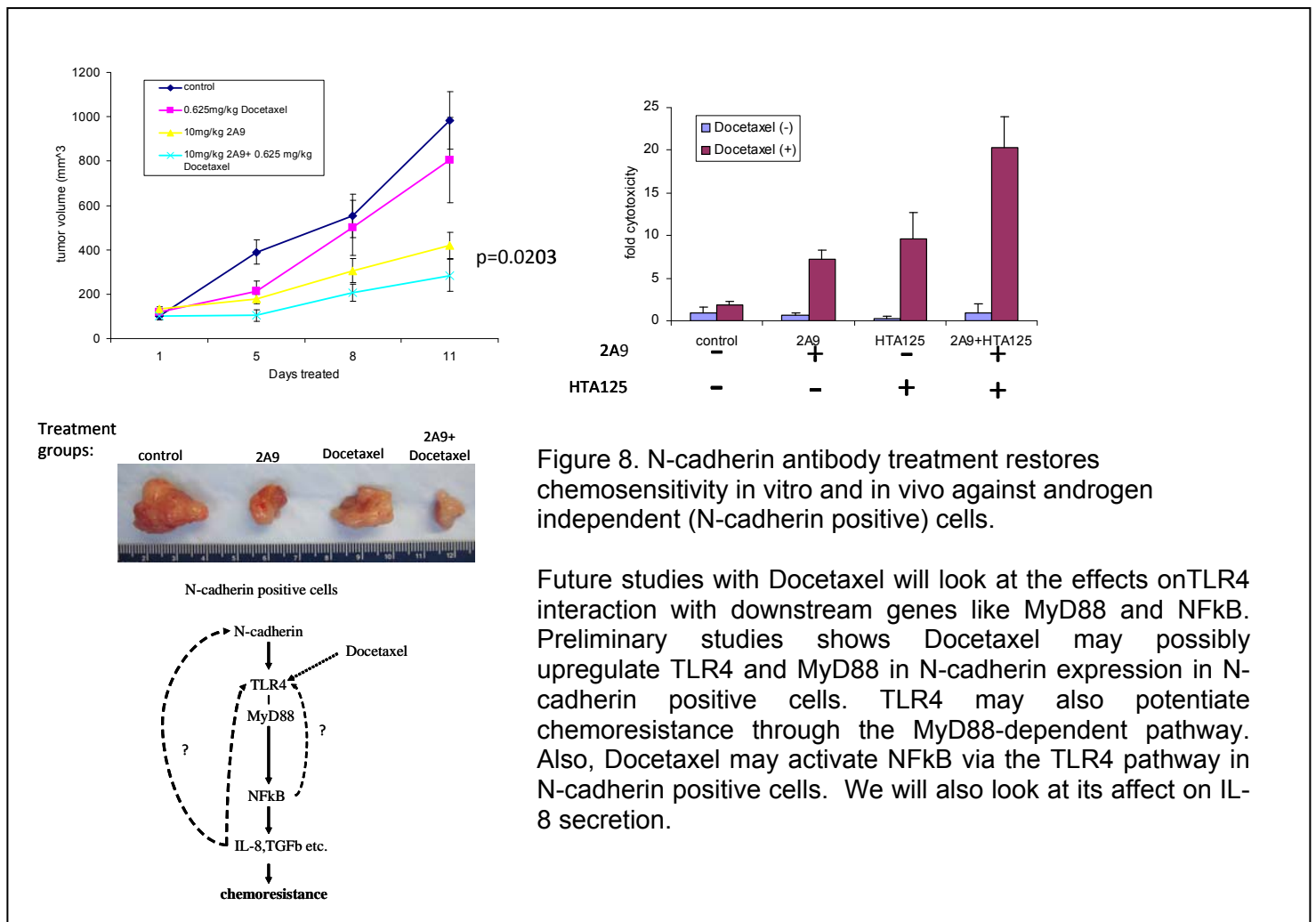
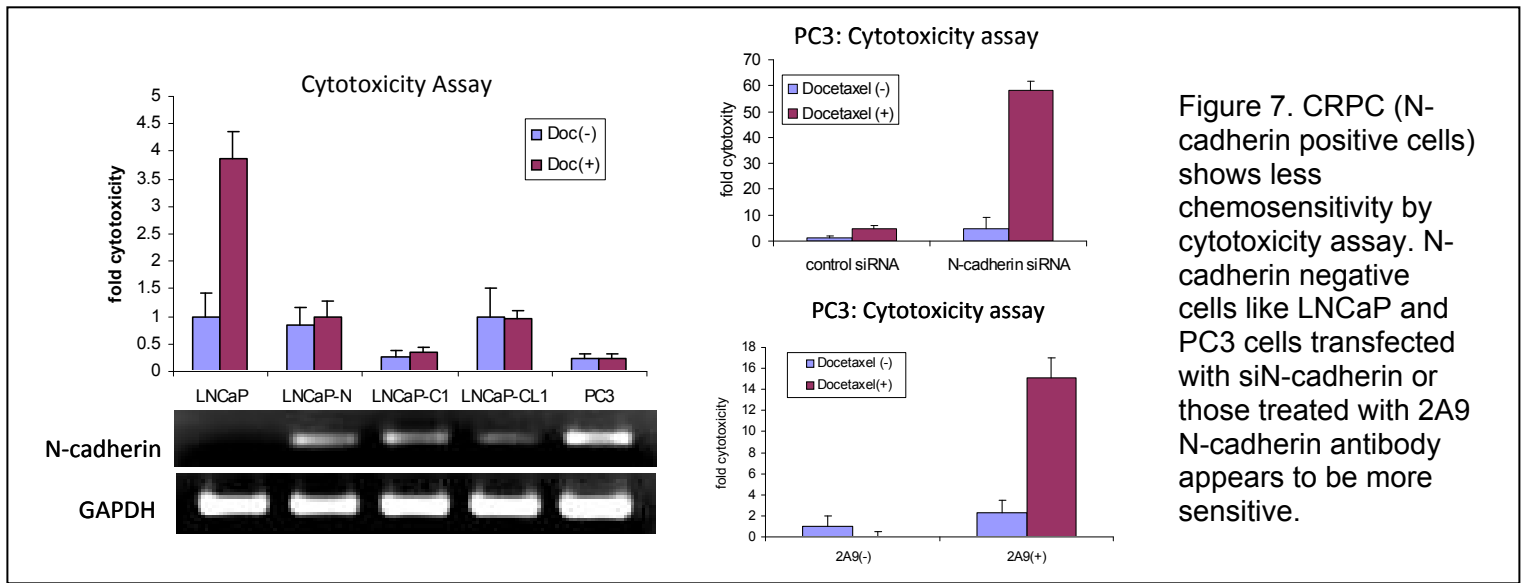


Treatment of LAPC-9CR passage 5 expressing high N-cadherin and very low AR shows resistance to MDV3100

## LNCaP-N: 1uM MDV3100



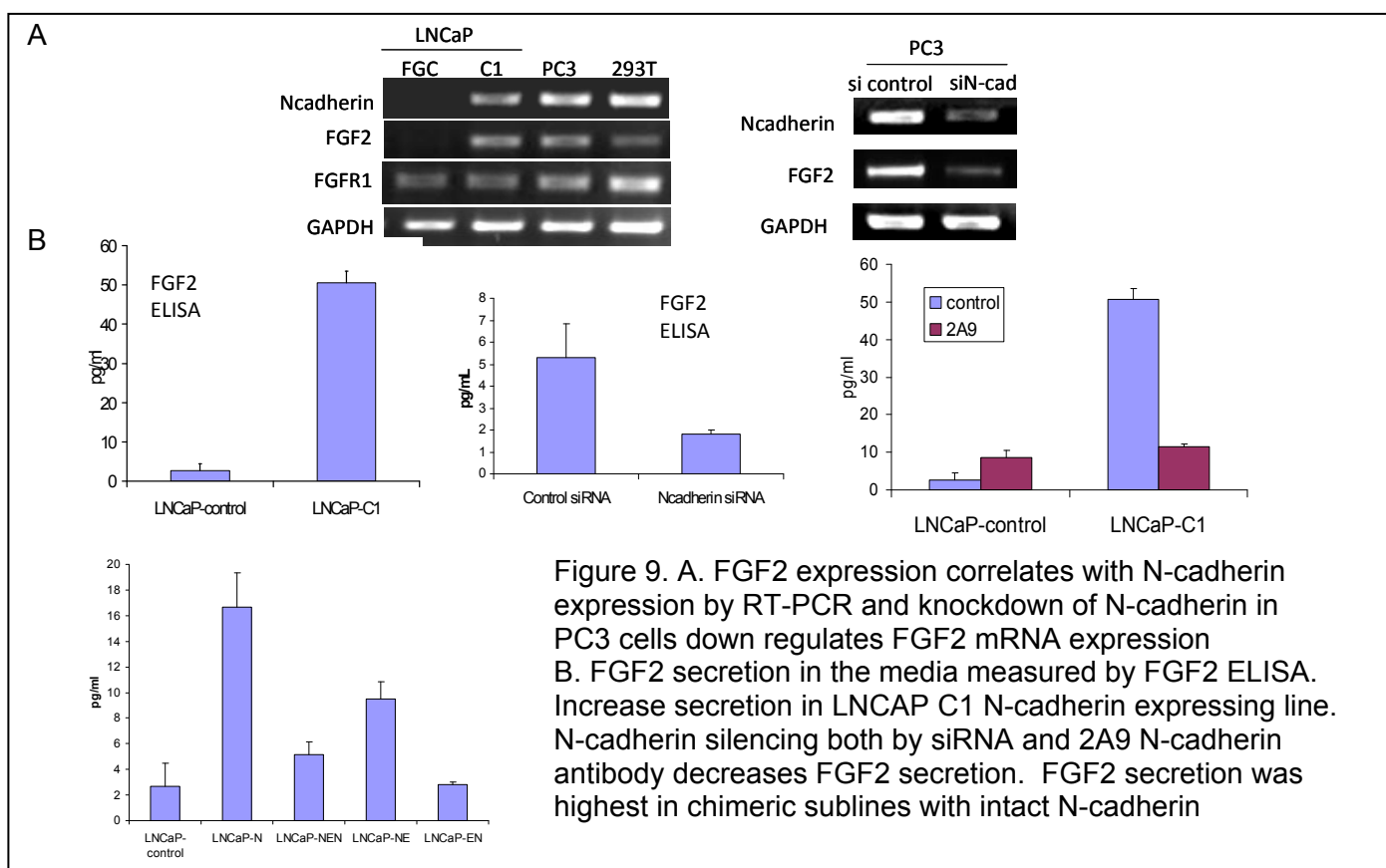
LNCaP-N cell proliferation in normal media: MDV3100 and 2A9 combination decreased cell proliferation.

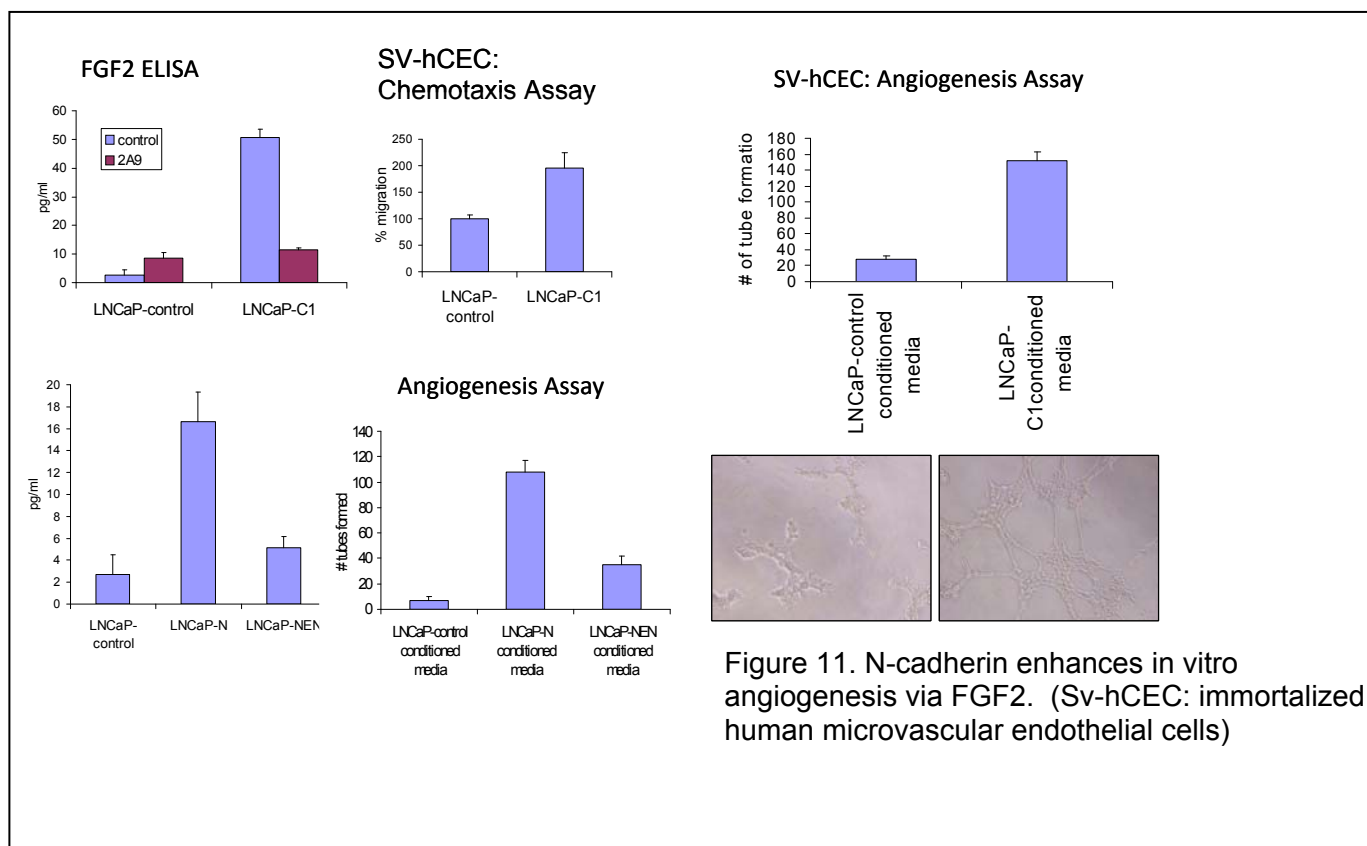
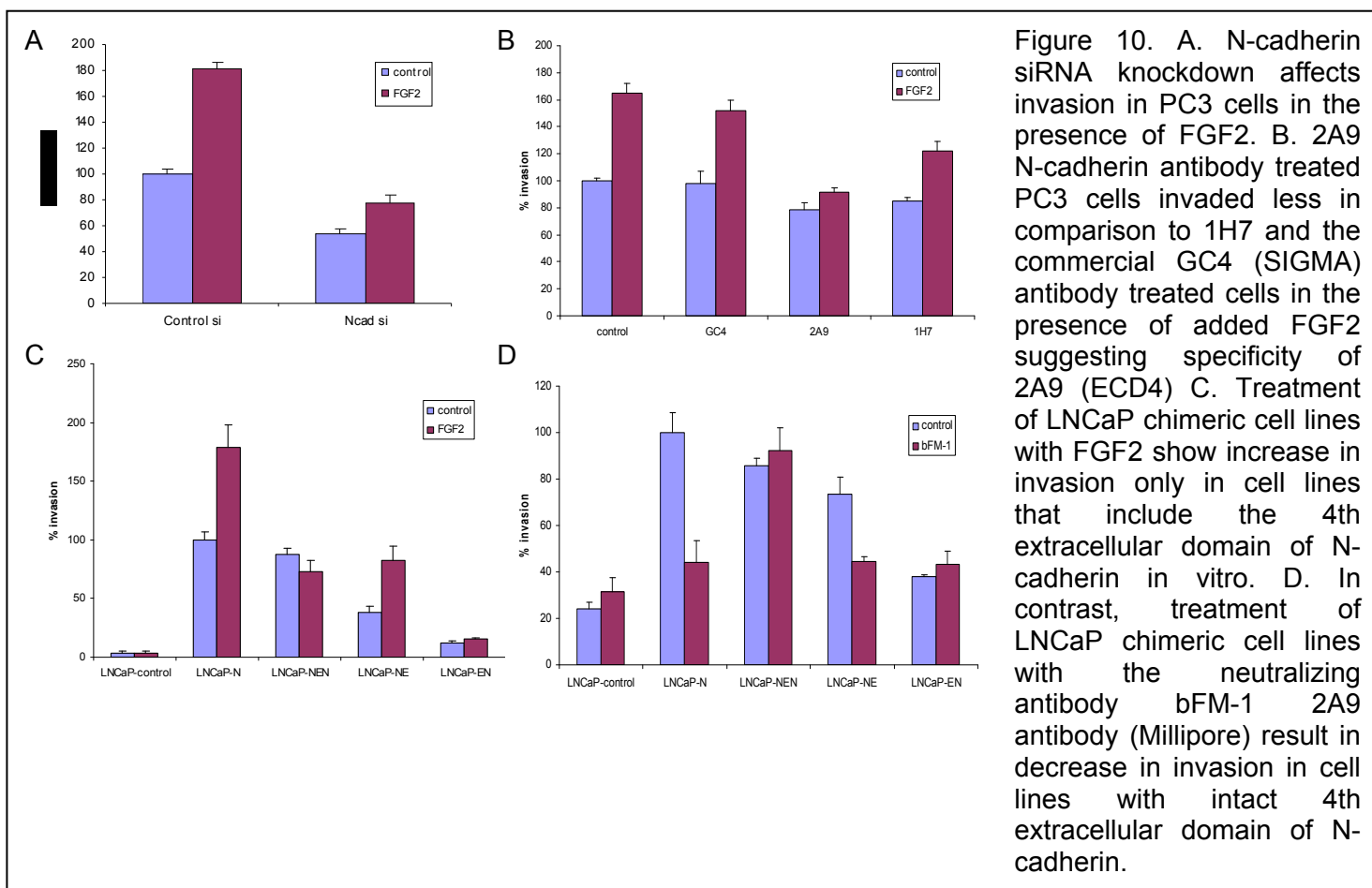


### 3.4 Biomarkers

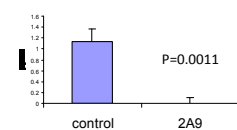
Biomarkers relevant to the pathways of interest (NF- $\kappa$ B, EMT, P13K) were assessed by Western, PCR, and serum ELISA for secreted proteins that might be useful in looking at activity of N-cadherin targeted therapy. Candidate secreted genes confirmed to be upregulated in microarrays by N-cadherin include IL-8, IL-6, FGF-2, TGF- $\beta$ , IL-15, TNF, VEGF and a number of chemokines. Interestingly, many of these, such as IL-8, have previously been implicated in castrate resistant progression. To date, we have confirmed that IL-8, IL-6, FGF-2 and TGF- $\beta$  are reduced by N-cadherin knockdown, indicating that they are N-cadherin targets and functionally important to N-cadherin biology.

Fibroblast growth factor 2 (FGF2) has been implicated in promoting tumor progression and angiogenesis. FGF2 has been reported to synergize with N-cadherin to enhance migration and invasion in breast cancer and other cancers. Studies suggest that the relationship between N-cadherin and FGF2 is involved in prostate cancer progression and is suggested that this relationship entails, at least in part, the 4th cellular domain of N-cadherin. (Figures 9, 10, 11, and 12)

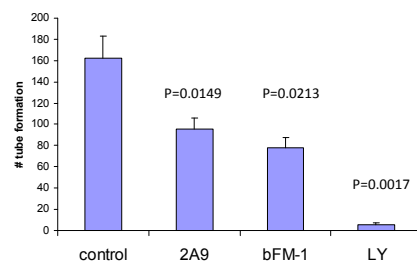




### PC3 FGF2 ELISA



### SV-hCEC: Angiogenesis Assay



### SV-hCEC: chemotaxis assay

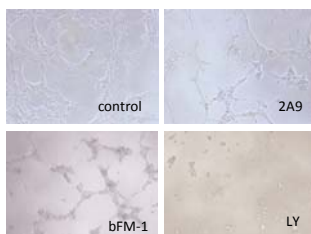
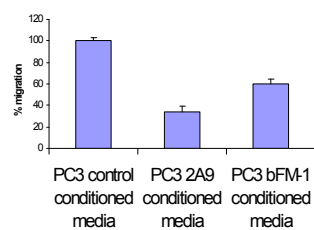


Figure 12. N-cadherin antibody 2A9 inhibits FGF2-induced in vitro angiogenesis

## SUMMARY of KEY RESEARCH ACCOMPLISHMENTS

### Yr 1:

- NF- $\kappa$ B activation is increased in N-cadherin positive cells.
- NF- $\kappa$ B inhibition reduced N-cadherin driven invasiveness.
- N-cadherin and AR expression are inversely correlated.
- N-cadherin expression induces activation of AKT and inhibition of PI3K/AKT in N-cadherin expressing cells results in specific reduction in NF- $\kappa$ B activation.
- Successfully generated NEmyc, NENmyc, ENmyc overexpressing cell lines, as well as control cell lines (empty vector, Ncad-myc, Ecad-myc).
- The N-cadherin ECD is required for cell mobility and invasion, while the intracellular domain is dispensable for motility.
- Ncad-myc subline showed enhanced in vitro castrate resistant growth compared to control or the chimeric sublines.

### Yr 2:

- N-cadherin targeting monoclonal antibody or PI3K inhibitor reduced N-cadherin driven invasiveness, and combination treatment showed synergy in vitro.
- N-cadherin antibody or PI3K inhibitor reduced TGF $\beta$ -induced invasiveness, and combination treatment showed synergy in vitro.
- N-cadherin antibody alone did not reduce in vitro cell proliferation at time point when IKK inhibitor was effective, and combination treatment showed no synergy.
- N-cadherin antibody alone reduced EGF-induced in vitro cell proliferation, but PI3K inhibitor was more effective and there was no synergy in combination treatment.
- N-cadherin overexpression in AR positive prostate cancer cells resulted in less inhibition of in vitro cell proliferation when treated with AR antagonist MDV3100.
- Combination of N-cadherin antibody and lower dose of MDV3100 showed synergistic effect in reducing in vitro cell proliferation.
- The N-cadherin extracellular domain (ECD) was required for cell motility, but ECD4 and intracellular domain (ICD) were dispensable.
- Both ECD and ICD were crucial for N-cadherin driven cell invasion, while ECD4 played a minor role.
- Both ECD and ICD and ECD4 were important for N-cadherin driven castrate resistant growth in vitro.
- Both ECD and ICD were required for N-cadherin driven CRPC growth in vivo, while ECD4 appeared to play a minor role.
- N-cadherin promoted CRPC growth without full transition from epithelial to mesenchymal phenotype.
- In vivo induction of vimentin expression required N-cadherin ECD, while ECD4 and ICD were dispensable.
- N-cadherin overexpression in AR positive prostate cancer cells resulted in increased AR expression under androgen-depleted condition.
- Combination of N-cadherin antibody and MDV3100 did not show synergy in CRPC tumor growth inhibition, but further dose optimization is needed.

### Yr 3:

- Correlation between N-cadherin expression and enhanced phosphorylation of SRC (Tyr416), involved in RTK signaling (FGFR)
- TLR4 gene expression is up-regulated in N-cadherin positive prostate cell lines
- Treatment of LAPC-9CR passage 3 expressing both N-cadherin and AR with MDV3100 shows partial sensitivity. Treatment of LAPC-9CR passage 5 expressing high N-cadherin and very low AR shows resistance to MDV3100
- N-cadherin antibody treatment restores chemosensitivity in vitro and in vivo against androgen independent (N-cadherin positive) cells
- FGF2 expression correlates with N-cadherin expression. FGF2 media secretions increase in N-cadherin overexpressing cell lines and decrease when treated with neutralizing antibody or siRNA.
- N-cadherin enhances in vitro angiogenesis via FGF2. N-cadherin antibody inhibits FGF2 induced angiogenesis

## **REPORTABLE OUTCOMES**

Yr 1:

- Manuscript in press in Nature Medicine (appendices).
- Multli-PI R01 submitted to NIH for production/humanization of our Ncadherin monoclonal antibody (decision on funding for percentile of 17% - did not get funded).

Yr 2:

- No reportable outcomes

Yr 3:

- In the process of writing two manuscripts based on the results of Aim 2 and Aim 3 (Chemoresistance and FGF2 data).

## CONCLUSION

Yr 1:

Our results suggested that in N-cadherin driven advanced prostate cancer, inhibition of downstream signaling pathways especially NF- $\kappa$ B and the PI3K/AKT pathways may serve as complementary therapies to N-cadherin targeting. Furthermore, we showed that the N-cadherin extracellular, but not intracellular, domain is crucial for prostate cancer cell motility and possibly invasion. Intact full-length N-cadherin is required to confer castrate resistant growth.

Yr 2:

The *in vivo* experiments using the chimeric cadherin sublines showed that intact Ncadherin was required for N-cadherin driven CRPC growth in vivo, while ECD4 appeared to play a minor role. However using either our 2A9 antibody which recognizes ECD4, or the 1H7 antibody which recognizes ECD1-3, we have shown effective CRPC tumor inhibition. We were able to generate three of the four intended chimeric cadherin overexpressing cell lines. Furthermore, we showed initial evidence that intact full-length N-cadherin is required to confer growth advantage in androgen depleted medium. This implied that N-cadherin signaling events in castrate resistant growth might be distinct from cell invasion.

Yr 3:

N-cadherin expression may be an adaptive response to castration that is sufficient enough to cause progression to castration resistance and may be also a cause of treatment resistance as seen with MDV3100 occurring in later passage CRPC LAPC-9 with low AR expression. Genes involved in signaling pathways implicating EMT are highly expressed including members of MAPK (FGFR, FGF-2) and NF- $\kappa$ B (TLR4) pathways. Treatment of N-cadherin expressing cells with specific inhibitors, antibodies, or siRNA, reduced invasion, angiogenesis, chemoresistance, proliferation, etc. Our data demonstrates the need for multiple targets for treatment and therapy.

## REFERENCES

- [1] Tanaka H, Kono E, Tran CP, Miyazaki H, Yamashiro J, Shimomura T, Ladan F, Wada R, Huang J, Vessella RL, An J, Horvath S, Gleave M, Rettig MB, Wainberg ZA, Reiter RE. Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance. *Nat. Med.* 16(12):1414-20, 2010.
- [2] Yang SY, Miah A, Pabari A, Winslet M. Growth Factors and their receptors in cancer metastases. *Frontiers in Bioscience* 16: 531-538, 2011.
- [3] Tian M, Neil JR, Schiemann WP. Transforming growth factor- $\beta$  and the hallmarks of cancer. *Cell Signal* 23(6):951-62, 2011.
- [4] Kim JB, Islam S, Kim YJ, Prudoff RS, Sass KM, Wheelock MJ, Johnson KR. N-cadherin extracellular repeat 4 mediates epithelial to mesenchymal transition and increased motility. *J. Cell Biol.* 151(6): 1193-1205, 2000.
- [5] Roudier MP, True LD, Higano CS, Vessella H, Ellis W, Lange P, et al. Phenotypic heterogeneity of endstage prostate carcinoma metastatic to bone. *Hum Pathol.* 2003;34(7):646-53. PubMed PMID: 12874759.
- [6] Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* 2004;64(24):9209-16. PubMed PMID: 15604294.

# Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance

Hiroshi Tanaka<sup>1,2,12</sup>, Evelyn Kono<sup>1,2,12</sup>, Chau P Tran<sup>1,2</sup>, Hideyo Miyazaki<sup>3</sup>, Joyce Yamashiro<sup>1,2</sup>, Tatsuya Shimomura<sup>1,2</sup>, Ladan Fazli<sup>4</sup>, Robert Wada<sup>1</sup>, Jiaoti Huang<sup>2,5</sup>, Robert L Vessella<sup>6</sup>, Jaibin An<sup>1,7</sup>, Steven Horvath<sup>8,9</sup>, Martin Gleave<sup>4</sup>, Matthew B Rettig<sup>1,2,7</sup>, Zev A Wainberg<sup>2,10</sup> & Robert E Reiter<sup>1,2,11</sup>

The transition from androgen-dependent to castration-resistant prostate cancer (CRPC) is a lethal event of uncertain molecular etiology. Comparing gene expression in isogenic androgen-dependent and CRPC xenografts, we found a reproducible increase in N-cadherin expression, which was also elevated in primary and metastatic tumors of individuals with CRPC. Ectopic expression of N-cadherin in nonmetastatic, androgen-dependent prostate cancer models caused castration resistance, invasion and metastasis. Monoclonal antibodies against the ectodomain of N-cadherin reduced proliferation, adhesion and invasion of prostate cancer cells *in vitro*. *In vivo*, these antibodies slowed the growth of multiple established CRPC xenografts, blocked local invasion and metastasis and, at higher doses, led to complete regression. N-cadherin-specific antibodies markedly delayed the time to emergence of castration resistance, markedly affected tumor histology and angiogenesis, and reduced both AKT serine-threonine kinase activity and serum interleukin-8 (IL-8) secretion. These data indicate that N-cadherin is a major cause of both prostate cancer metastasis and castration resistance. Therapeutic targeting of this factor with monoclonal antibodies may have considerable clinical benefit.

Men with prostate cancer die predominantly from metastatic disease that is resistant to androgen deprivation therapy. Although the complete cause of castration resistance is not known, recent studies indicate that a large percentage of castration-resistant tumors progress by maintaining androgen receptor-dependent signaling. Mechanisms underlying the preservation of androgen receptor signaling include androgen receptor overexpression, growth factor-regulated androgen receptor activation and *de novo* intracrine androgen production<sup>1–4</sup>. New treatments designed to block androgen receptor activity (MDV3100) and steroidal synthesis (for example, abiraterone or TAK-700) have entered the clinic with promising preliminary results.

Despite these advances, it is not certain that androgen receptor reactivation is the only cause of castration resistance or that abrogation of androgen receptor signaling will result in cure. Lethal prostate cancers are heterogeneous, with pockets of cells that overexpress androgen receptor and others that do not express detectable androgen receptor<sup>5,6</sup>. Initial results with the newest androgen receptor-targeted drugs are extremely promising, but early data suggest that 30% of patients do not respond at all, and 30–40% have only partial responses<sup>7,8</sup>. The mechanisms by which tumors resist newer antiandrogens are

not known, but the existence of tumors that are resistant to these approaches suggests that some tumors may be androgen receptor independent or only partially androgen receptor dependent.

There are a number of potential androgen receptor-independent mechanisms of castration resistance. For example, castration induces multiple antiapoptotic genes<sup>9,10</sup>. Recent clinical studies of agents that block these pathways have had initial promise. There has also been a surge of interest in the role of prostate cancer stem cells in prostate cancer development and progression<sup>11,12</sup>. Although controversial, some studies suggest that normal and prostate cancer stem cells may not express androgen receptor, implying that prostate cancers may become castration resistant through survival and expansion of cancer-initiating cells that lack functional androgen receptor.

To identify alternative pathways of castration resistance, we compared gene expression in matched androgen-dependent and CRPC xenografts. N-cadherin, a mesenchymal cadherin associated with epithelial-to-mesenchymal transition (EMT), was reproducibly upregulated in several models of castration-resistant cancer. We validated the association of N-cadherin with castration resistance in clinical samples of CRPC. These findings prompted us to perform a series

<sup>1</sup>Department of Urology, Geffen School of Medicine, University of California–Los Angeles, Los Angeles, California, USA. <sup>2</sup>Jonsson Comprehensive Cancer Center, Geffen School of Medicine, University of California–Los Angeles, Los Angeles, California, USA. <sup>3</sup>Department of Urology, University of Tokyo, Tokyo, Japan. <sup>4</sup>Vancouver Prostate Center, University of British Columbia, Vancouver, British Columbia, Canada. <sup>5</sup>Department of Pathology, Geffen School of Medicine, University of California–Los Angeles, Los Angeles, California, USA. <sup>6</sup>Department of Urology, University of Washington Medical Center, Puget Sound Veterans Health Care Administration, Seattle, Washington, USA. <sup>7</sup>Department of Medicine, Veterans Affairs Greater Los Angeles Healthcare System–West Los Angeles, Los Angeles, California, USA. <sup>8</sup>Department of Human Genetics, David Geffen School of Medicine, University of California–Los Angeles, Los Angeles, California, USA. <sup>9</sup>Department of Biostatistics, School of Public Health, University of California–Los Angeles, Los Angeles, California, USA. <sup>10</sup>Division of Hematology and Oncology, Geffen School of Medicine, University of California–Los Angeles, Los Angeles, California, USA. <sup>11</sup>Molecular Biology Institute, Geffen School of Medicine, University of California–Los Angeles, Los Angeles, California, USA. <sup>12</sup>These authors contributed equally to this work. Correspondence should be addressed to R.E.R. (reiter@mednet.ucla.edu).

Received 16 April; accepted 9 September; published online 7 November 2010; doi:10.1038/nm.2236

**Figure 1** N-cadherin is upregulated in castration resistant prostate cancer. **(a)** N-cadherin and androgen receptor expression in multiple independently derived paired AD and CR LAPC4 and LAPC9 xenografts. **(b)** Protein expression of N-cadherin and E-cadherin in prostate cancer cell lines (LNCaP, PC3, 22RV1, LAPC9-AD and LAPC9-CR) and control cells (bladder cancer cell lines J82 and 647V). **(c)** FACS analysis of N-cadherin in serial passages (p) of LAPC9 from AD to CR. **(d)** Protein expression of N-cadherin, E-cadherin and AR in serial passages of LAPC9 from AD to CR. **(e)** Real-time PCR analysis of N-cadherin expression in multiple prostate cancer metastases (9, 15, 20, 22 and 23 are higher by more than 1,500-fold). Normalized expression (against glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) is shown as fold-change of LNCaP expression, with PC3 and LAPC9 included for comparison. **(f)** N-cadherin immunohistochemistry of high-expression prostate cancer metastases (M), showing clear staining in M1, M2 and M3 and no staining in AD. Scale bar, 500  $\mu$ m.

of *in vitro* and *in vivo* studies, with the hypothesis that N-cadherin is crucial in prostate cancer progression not only to metastasis, but also to castration resistance. Because N-cadherin is expressed on the cell surface, we also asked whether therapeutic targeting with N-cadherin-specific monoclonal antibodies would have efficacy in preclinical models. The major findings of our study are that N-cadherin expression is sufficient to cause invasive, metastatic and castration-resistant prostate cancer and that these effects can be inhibited by N-cadherin-specific antibodies. Furthermore, N-cadherin-specific antibodies can inhibit the growth of both androgen receptor-positive and androgen receptor-negative prostate cancers. These studies identify a previously unknown pathway responsible for metastasis and castration resistance and validate N-cadherin as a promising new target for prostate cancer treatment.

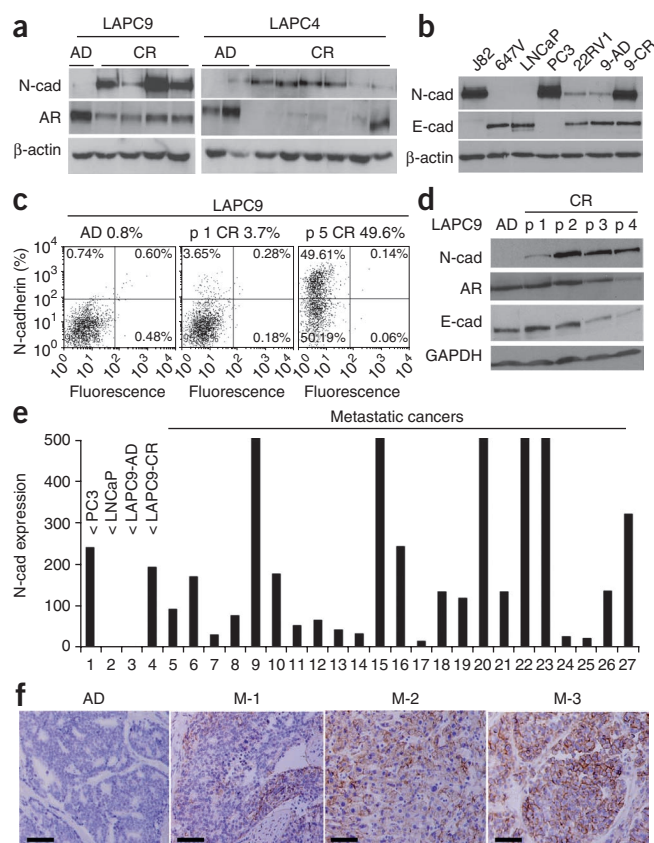
## RESULTS

### N-cadherin is upregulated in CRPC

To identify markers of castration resistance, we compared gene expression in paired hormone-sensitive (AD) and castration-resistant (CR) LAPC9 xenografts<sup>13</sup>. N-cadherin expression was highly elevated in LAPC9-CR xenografts<sup>13</sup>, which we confirmed by further screening of independently derived LAPC4 and LAPC9 xenografts (**Fig. 1a**). N-cadherin was absent in hormone-sensitive LNCaP but present in castration-resistant 22RV1, PC3 and LNCaP-CL1<sup>14</sup> prostate cancer cell lines (**Fig. 1b**). These data suggest that expression of N-cadherin is a common event in CRPC progression.

Next, we evaluated the kinetics of N-cadherin expression in serial passages of LAPC9-CR tumors in castrated mice. We detected N-cadherin in 1–5% of cells in tumors after the first passage, but it was present in 50% of cells by passage 5 (**Fig. 1c**), concomitant with gradual loss of E-cadherin and androgen receptor expression (**Fig. 1d**). These results suggest that N-cadherin-positive cells may have a growth advantage over N-cadherin-negative cells in castrated mice and that N-cadherin may be involved in the modulation of E-cadherin and androgen receptor expression.

To determine whether N-cadherin is expressed in clinical CRPC, we performed quantitative PCR and immunohistochemistry on 21 soft-tissue and bone metastases obtained from men who died from prostate cancer. N-cadherin was expressed in 16 of 21 metastases (**Fig. 1e**). Immunohistochemical staining confirmed N-cadherin protein expression in cases with high N-cadherin mRNA levels (**Fig. 1f**) and in three of six additional CRPC bone metastases. We also stained three tissue microarrays containing samples from individuals with benign prostatic hyperplasia, hormone-naïve prostate cancer, prostate cancer treated with 3–9 months of neoadjuvant



hormone ablation, and CRPC. We detected N-cadherin expression in 16.7%, 28%, 34% and 67% of these samples, respectively. The mean percentage of cells staining positive for N-cadherin among all samples increased from 1% in benign prostatic hyperplasia to 9.5% in hormone-naïve disease, 22.5% in men treated with neoadjuvant androgen deprivation and 41% in CRPC ( $P < 0.01$ ) (**Supplementary Fig. 1**). These data demonstrate that N-cadherin expression is rare in untreated androgen-dependent prostate cancer, increases with androgen deprivation and is highest in CRPC.

### N-cadherin causes invasion, metastasis and castration resistance

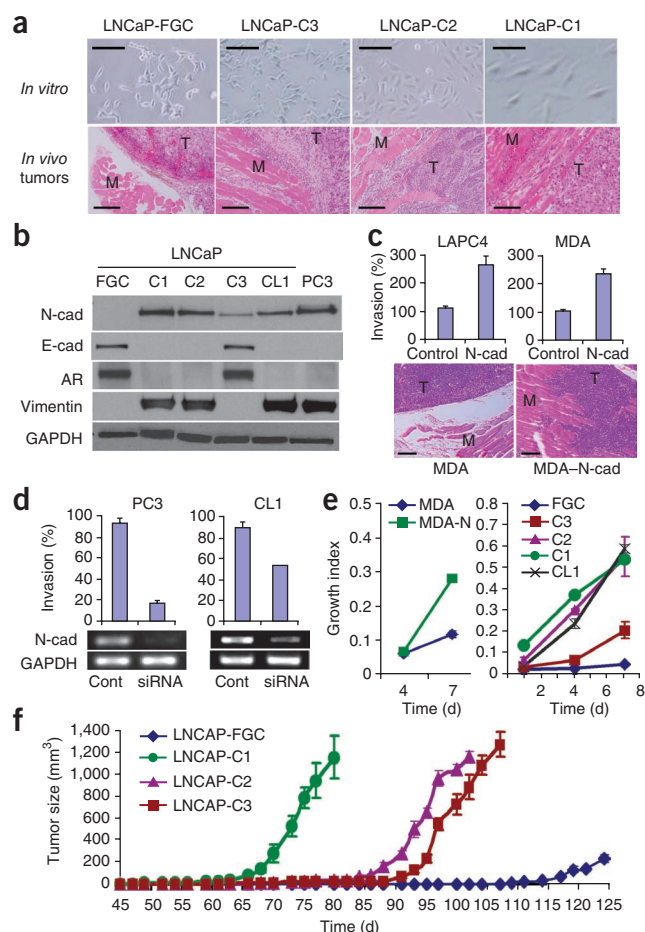
To evaluate the role of N-cadherin in prostate cancer, we ectopically expressed N-cadherin in multiple AD cell lines (LNCaP, MDA-PCa-2b and LAPC4). N-cadherin-positive cells appeared flattened and fibroblastic, concomitant with loss of E-cadherin and gain of vimentin, although one low-expressing LNCaP subline (C3) retained E-cadherin and did not change morphologically (**Fig. 2a,b**). All N-cadherin-expressing cell lines (including C3) became more invasive (**Fig. 2c**), and invasiveness correlated with N-cadherin abundance, indicative of a gene dosage effect. When implanted subcutaneously, N-cadherin-positive tumors invaded underlying muscle and spread to distant lymph nodes (**Fig. 2a,c**). Conversely, silencing N-cadherin in castration-resistant PC3 and CL1 cells reduced invasiveness (**Fig. 2d**). These data suggest that N-cadherin expression is sufficient to cause EMT, invasion and metastasis in prostate cancer cells.

The association of N-cadherin with CRPC suggested that it might have a role in castration resistance. Consistent with this hypothesis, N-cadherin-expressing cell lines (MDA-N and LNCaP-C1, LNCa-C2 and LNCa-C3) could proliferate in the absence of androgen *in vitro* (**Fig. 2e**). Most importantly, N-cadherin expression

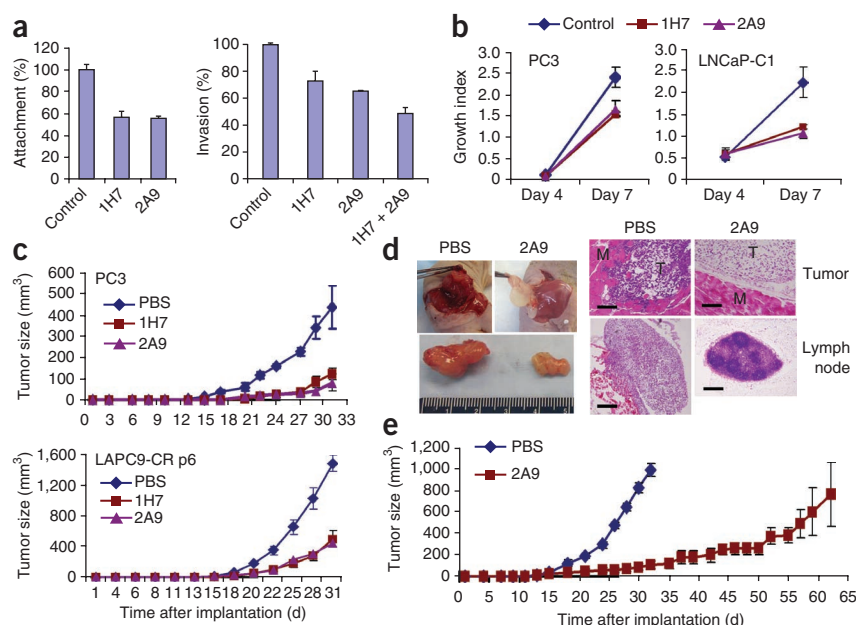
**Figure 2** N-cadherin causes invasion, migration and EMT of multiple prostate cancer cell lines. **(a)** Top, *in vitro* morphologic changes in LNCaP sublines that overexpress increasing amounts of N-cadherin (LNCaP-C3 < LNCaP-C2 < LNCaP-C1) compared to control cell line LNCaP-FGC (control). Scale bar, 50  $\mu$ m. Bottom, *in vivo* invasive tumor growth of LNCaP sublines in castrated mice, compared to control noninvasive tumor in intact mice. M, muscle; T, tumor. Scale bar, 500  $\mu$ m. **(b)** Western blot of N-cadherin-overexpressing sublines, showing loss of E-cadherin and androgen receptor, with gain of vimentin expression in C2 and C1. CL1 and PC3 are castration-resistant cell lines with endogenous N-cadherin. **(c)** Top, invasion assays in androgen-dependent LAPC4 ( $P = 0.009$ ) and MDA-Pca-2b ( $P = 0.016$ ) cells ectopically overexpressing N-cadherin. Bottom, deep muscle invasion of *in vivo* MDA-N-cadherin tumor versus noninvasive MDA tumor (control). Scale bar, 100  $\mu$ m. **(d)** Invasion assays in endogenous PC3 and CL1 cells upon N-cadherin silencing by siRNA silencing,  $P = 0.003$ . Cont, control (scrambled siRNA). **(e)** *In vitro* castration-resistant growth of both MDA-PCa-2b and LNCaP sublines overexpressing N-cadherin ( $P = 0.014$  versus FGC), C3 versus FGC ( $P = 0.029$ ). **(f)** *In vivo* castration-resistant growth of LNCaP-FGC, C1, C2, and C3 when implanted in castrated mice. Data are shown as means  $\pm$  s.e.m.

conferred castration resistance *in vivo*, as evidenced by the ability of all N-cadherin-transduced cell lines to form tumors in castrated mice (Fig. 2f). Castration-resistant growth correlated with the level of N-cadherin expression, with LNCaP-C1 cells growing more rapidly than C2 and C3 cells *in vitro* and *in vivo* (Fig. 2e,f). To determine whether N-cadherin is required for castration resistance, we knocked it down in PC3 and LNCaP-CL1 cells. Stable silencing of N-cadherin significantly ( $P = 0.005$ ) impaired the ability of both cell lines to form tumors in castrated mice (Supplementary Fig. 2). These data suggest that N-cadherin expression is both sufficient and necessary for castration resistant growth.

N-cadherin expression led to an inverse loss of androgen receptor, with high expressors (LNCaP-C1, PC3 and CL1) losing androgen receptor completely and low-expressors (LNCaP-C3) retaining it (Fig. 1a and Fig. 2b). We saw a similar pattern in LAPC9-CR cells, with some cells expressing androgen receptor, some expressing N-cadherin and others expressing both androgen receptor and N-cadherin (Supplementary Fig. 3). These data indicate that



N-cadherin is sufficient to cause androgen receptor-independent prostate cancer. However, many prostate cancers coexpress N-cadherin and androgen receptor, suggesting that these factors may act synergistically to promote castration-resistant growth.



**Figure 3** Antibodies against N-cadherin decrease invasion and tumor growth. **(a)** Attachment ( $P = 0.004$ ) and invasion ( $P = 0.05$ ) assays of PC3 cells upon treatment without or with 1H7 or 2A9 (80  $\mu$ g ml<sup>-1</sup>). **(b)** Decrease in cell growth measured by proliferation assays of PC3 ( $P = 0.014$ ) and LNCaP-C1 ( $P = 0.01$ ) cells, upon treatment without or with 80  $\mu$ g ml<sup>-1</sup> 1H7 or 2A9. **(c)** *In vivo* castration-resistant growth inhibition (>70%) of both PC3 and LAPC9-CR (passage 6) tumors upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight, beginning when subcutaneous tumors were palpable in castrated mice.  $P = 0.016$  for both cell lines compared to control (PBS) group at 31 d. **(d)** Gross and histological analyses of mice treated without or with 2A9, showing decrease in tumor size, tumor-muscle invasion (scale bar, 200  $\mu$ m) and metastases to axillary lymph nodes (scale bar, 500  $\mu$ m). **(e)** Continuous growth inhibition of PC3 tumors upon long-term treatment with 2A9 antibody (50% at 57 d compared to control group at 32 days,  $P = 0.025$ ). Data are shown as means  $\pm$  s.e.m.

### N-cadherin antibodies inhibit growth of CRPC

The presence of N-cadherin in metastatic prostate cancer and its ability to promote castration resistance suggested that N-cadherin might be a therapeutic target in advanced prostate cancer. We generated a panel of monoclonal antibodies specific for the extracellular domain of N-cadherin to test this hypothesis and to determine which domains are necessary for its effects in prostate cancer. We screened antibodies for cell surface recognition of N-cadherin and an ability to inhibit invasion *in vitro*. We selected two antibodies: 1H7, a mouse IgG1, recognizes an epitope within the first three extracellular domains, whereas 2A9, an IgG2a, recognizes an epitope in the fourth domain. Both antibodies inhibited invasion, attachment and proliferation of PC3 and LNCaP-C1 cells *in vitro* (Fig. 3a,b). Upon exposure to either antibody, PC3 and LNCaP-C1 cells showed morphologic changes, such as increased polarity, resembling an epithelial phenotype (data not shown). These results suggest that N-cadherin-specific antibodies can affect multiple parameters of *in vitro* growth, including invasion, proliferation, attachment and potentially EMT.

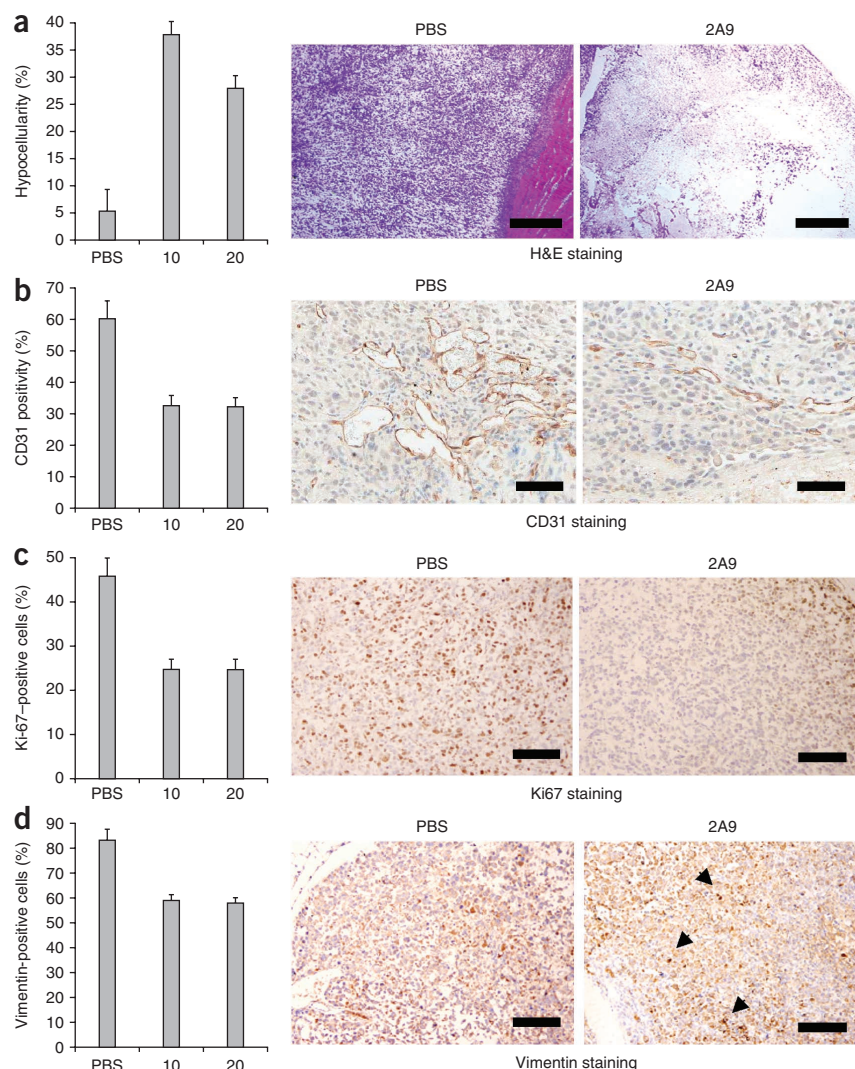
We next asked whether N-cadherin-specific antibodies could affect invasion, metastasis and castration-resistant tumor growth *in vivo*. Castrated mice bearing palpable PC3, LAPC9-CR and LNCaP-C1 tumors were treated twice weekly with PBS or the antibodies 1H7 or 2A9 (10 mg per kg body weight) for 2 weeks. Both antibodies inhibited tumor growth (Fig. 3c). The antibody-treated tumors were pale, nonadherent to underlying muscle, and noninvasive histologically, whereas control tumors grossly invaded underlying muscle (Fig. 3d). In addition, N-cadherin-specific antibody-treated mice had rare distant lymph node metastases (one out of five mice treated with 1H7, zero of five mice treated with 2A9), whereas 100% of nodes (five of five) were replaced by cancer in control mice (Fig. 3d). Prolonged administration of 2A9 led to long-term growth suppression and a >100% mean improvement in survival of mice bearing PC3 tumors (Fig. 3e). Treated tumors had large areas of cell loss, reduced proliferation (Ki-67 staining), fewer blood vessels (CD31 staining), less vimentin staining and lower N-cadherin expression compared to untreated tumors (Fig. 4 and Supplementary Fig. 4). These data indicate

that antibodies targeting the N-cadherin ectodomain are able to inhibit tumor growth, local invasion and metastasis of CRPC.

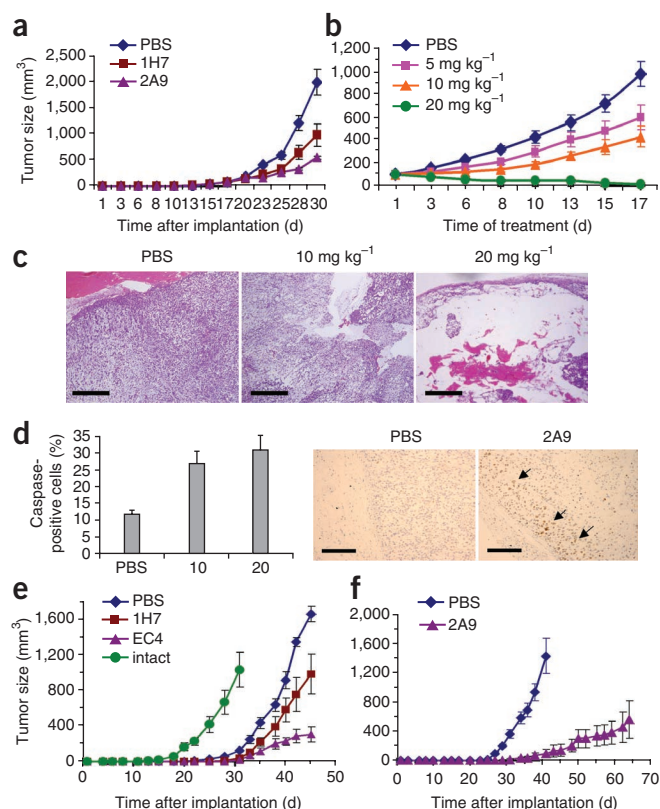
We also administered N-cadherin-specific antibodies to mice with larger established tumors. Both antibodies significantly slowed the growth of all three tumor models, although 2A9 suppressed growth better than 1H7 in most experiments (Fig. 5a). Dose escalation of 2A9 to 20 mg per kg body weight led to complete regression of >50% of PC3 tumors, whereas no additional benefit was seen with 40 mg per kg body weight (Fig. 5b). To examine the mechanism of tumor regression, we collected a subset of tumors within days of starting antibody treatment (Fig. 5c). We saw large areas of cell loss and necrosis in treated tumors, as well as more caspase-3 staining (Fig. 5d), suggesting that apoptosis may temporally precede the cell loss seen after prolonged treatment. These data show that N-cadherin-specific antibodies can suppress the growth of large established tumors and that higher doses can cause tumor regression.

### N-cadherin antibodies delay progression to castration resistance

To test the requirement for N-cadherin in castration-resistant progression, we implanted LAPC9-AD tumors into castrated mice, treated them with N-cadherin-specific antibodies and monitored time to castration-resistant growth. Treatment with 2A9 significantly delayed time to castration resistance, whereas 1H7 only briefly delayed tumor



**Figure 4** Histological and immunohistochemical assessments of PC3 tumors treated without or with 2A9 at 10 or 20 mg per kg body weight. (a–d) Quantification of changes between untreated and treated tumors in the following parameters: hypocellular regions ( $P = 0.004$ ) by H&E staining (scale bar, 1.0 mm) (a); CD31 staining ( $P < 0.001$ ; scale bar, 500  $\mu$ m) (b); Ki-67 staining ( $P = 0.002$ ; scale bar, 500  $\mu$ m) (c); vimentin-positive regions (20 mg per kg body weight dose,  $P = 0.032$ ) (d). The arrowhead points to stained cells. Scale bar, 500  $\mu$ m. Quantification was determined by counting five different fields per tumor, followed by averaging the values for the five tumors. Data are shown as means  $\pm$  s.e.m.



**Figure 5** N-cadherin antibodies inhibit growth of established tumor and block progression to castration resistance *in vivo*. (a) Growth inhibition of established LAPC9-CR tumors (100 mm<sup>3</sup>) upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight.  $P = 0.003$  for both antibodies compared to control (PBS) group at 30 d. (b) Growth inhibition of established PC3 tumors (100 mm<sup>3</sup>) upon treatment without or with escalating doses of 2A9, starting at 5 mg per kg body weight.  $P = 0.024$  compared to control (PBS) group at 17 d. (c) Histology of untreated versus antibody-treated tumors. Scale bar, 1.0 mm. (d) Caspase-3 staining in untreated versus antibody-treated tumors at both 10 and 20 mg per kg body weight doses ( $P < 0.005$ ). Arrow, stained cells. Scale bar, 500  $\mu$ m. (e) Delay of LAPC9-CR tumor emergence upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight.  $P = 0.023$  compared to control (PBS) group at 45 d. Intact, mice bearing LAPC9-AD tumors without castration. (f) Same experiment as in e but with continuous 2A9 treatment, showing prolonged suppression of CR tumor growth after progression to castration resistance. Data are shown as means  $\pm$  s.e.m.

growth (Fig. 5e,f). Of note, only ~4% of cells in the untreated control tumors expressed N-cadherin, consistent with previous experiments showing that N-cadherin is expressed by a minority of cells in early-stage CR tumors (Fig. 1c). These data suggest that N-cadherin is required for the development of castration resistance and that therapeutic targeting of N-cadherin in AD tumors (even in a minority of cells) can markedly delay the emergence of CRPC.

### N-cadherin alters expression of genes implicated in CRPC

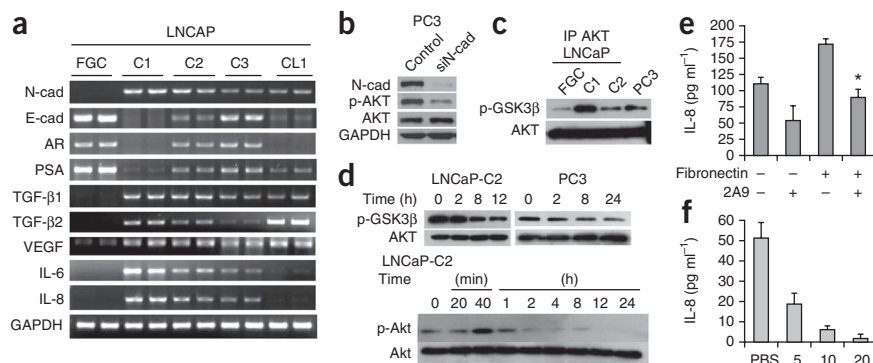
To gain insight into the mechanism of N-cadherin activity in prostate cancer, we compared the expression profiles of N-cadherin-transduced cells and controls. We selected genes previously shown to be associated with progression of LNCaP cells to the castration-resistant LNCaP-CL1 subline as a starting point<sup>14</sup>. As predicted, N-cadherin-transduced cells showed the characteristic changes of an EMT, with decreased E-cadherin expression (Fig. 6a) and increased vimentin expression (data not shown). These changes were proportional to the level of N-cadherin expression. Other notable changes included increased B cell lymphoma-2 (bcl-2) expression (data not shown), increased transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), TGF- $\beta$ 2 and vascular endothelial growth factor (VEGF) expression, reduced androgen receptor and prostate-specific antigen expression and increased IL-6 and IL-8 expression (Fig. 6a). The loss of androgen receptor is consistent with our observation in LAPC-9 cells that N-cadherin expression is inversely correlated

with androgen receptor expression. Bcl-2 expression may explain the ability of N-cadherin-positive cells to survive in an androgen-depleted environment<sup>15</sup>. TGF- $\beta$  can induce EMT and might mediate N-cadherin signal transduction. TGF- $\beta$ , IL-6 and IL-8 have all previously been implicated in CRPC<sup>16,17</sup>. Silencing of N-cadherin in PC3 cells decreased IL-6, IL-8, vimentin, TGF- $\beta$  and VEGF expression but did not restore androgen receptor or E-cadherin expression, suggesting that more prolonged knockdown might be required for complete reversal of EMT (Supplementary Fig. 5).

Previous studies have associated N-cadherin with phosphoinositide 3-kinase-AKT pathway activation<sup>15</sup>. N-cadherin silencing reduced AKT phosphorylation, whereas N-cadherin overexpression correlated with increased AKT activity (Fig. 6b,c). These results indicate that N-cadherin is sufficient to cause EMT and regulates the expression of multiple genes implicated in castration resistance.

### N-cadherinantibody reduces AKT activity and IL-8 secretion

To determine the effects of N-cadherin-targeting antibody treatment on gene expression, we exposed PC3 and LNCaP-C2 cells *in vitro* to 2A9 and determined whether 2A9 reduced AKT kinase activity and IL-8 production. 2A9 reduced both phospho-AKT abundance and AKT kinase activity over a 4- to 24-h time period (Fig. 6d). ELISA of cell culture media after 2A9 treatment showed a >50% reduction



**Figure 6** Gene expression change in N-cadherin-overexpressing cells. (a) RT-PCR analyses of gene expression in LNCaP cells without (FGC) or with high (C1, C2 and CL1) and low (C3) levels of N-cadherin. (b) Western blot of PC3 cells upon N-cadherin siRNA silencing (siN-cad). (c) AKT kinase activity in N-cadherin-overexpressing cell lines, measured by *in vitro* kinase assay. (d) Changes in AKT kinase activity and phospho-AKT level in *in vitro* time-course treatment with 2A9 at 80  $\mu$ g ml<sup>-1</sup> in LNCaP-C2 or PC3 cells. (e) Changes in fibronectin-induced IL-8 secretion in cell media ( $*P = 0.027$ ) upon *in vitro* 2A9 treatment at 80  $\mu$ g ml<sup>-1</sup> in LNCaP-C1. (f) Changes in serum IL-8 level in PC3 tumor-bearing mice treated with 2A9 antibody at 5 ( $P = 0.014$ ), 10 and 20 mg per kg body weight ( $P < 0.001$ ). Data are shown as means  $\pm$  s.e.m.

in IL-8 secretion (Fig. 6e). 2A9 treatment also led to progressive declines in serum IL-8 that correlated with antibody dose and tumor regression (Fig. 6f). These data indicate that the N-cadherin-specific antibody 2A9 can reverse N-cadherin-induced activation of AKT and IL-8 expression and may explain, at least in part, the antitumor activity of this antibody. IL-8 could serve as a potential biomarker of N-cadherin and N-cadherin-targeted therapy.

## DISCUSSION

N-cadherin expression is reproducibly associated with progression to castration resistance in both LAPC4 and LAPC9 prostate cancer xenografts. N-cadherin is expressed in multiple CRPC cell lines and in a majority of metastatic and castration-resistant prostate cancer tissues. N-cadherin induction after neoadjuvant hormone ablation supports the association of this protein with castration resistance. Our findings differ somewhat from previous studies that have reported higher N-cadherin expression in high-risk primary tumors. For example, one group reported that N-cadherin was expressed in 50% of high-grade primary tumors and lymph node metastases<sup>18</sup> and in 65% of tumors with Gleason score of  $\geq 7$  (ref. 19). Another study showed that an E- to N-cadherin switch in primary tumors was predictive of recurrence and prostate cancer-related death<sup>20</sup>. Some of the differences between our results and those of these studies might be ascribed to technical issues such as antibody selection. Differences in the subject populations (that is, Europe versus US) might also explain the differences in reported expression between the studies. Regardless, our study and others confirm that N-cadherin is expressed in a considerable percentage of human prostate cancers and validate N-cadherin as a promising therapeutic target in this disease.

N-cadherin expression increases with passaging of castration-resistant tumors in our xenograft models, suggesting that N-cadherin-positive cells have a growth advantage over N-cadherin-negative cells and that a small percentage of N-cadherin-positive cells may be sufficient to drive castration resistance. Consistent with these hypotheses, N-cadherin-positive cells proliferate more rapidly than N-cadherin-negative cells. N-cadherin-positive cells from LAPC9-CR tumors are also more tumorigenic than N-cadherin-negative cells (E.K. and R.E.R., unpublished data). A number of recent studies have linked EMT and EMT-associated genes with cancer stem cells. Induction of EMT in immortalized mammary cells produced cells with stem cell properties such as mammosphere formation and tumorigenicity<sup>21</sup>. These studies raise the possibility that N-cadherin may be a marker for a population of castration-resistant stem cells in prostate cancer. This possibility is supported by our finding that N-cadherin-positive cells are tumorigenic and that antibody treatment was sufficient to delay progression to castration resistance, even though N-cadherin was only expressed by a small percentage of cells in the untreated controls. It is also supported by our finding that N-cadherin is expressed by only a fraction of cells in many human primary tumors, and this expression increases after androgen ablation and recurrence. Additionally, many stem cell-associated genes are upregulated in N-cadherin-positive cells (Supplementary Fig. 6)<sup>22</sup>. Additional studies will be required to establish whether N-cadherin-expressing cells are prostate cancer stem cells and whether they are required for castration-resistant or metastatic progression. Nevertheless, our data suggest that targeting of a small subset of cells with the potential to initiate castration-resistant tumor growth may be sufficient to have a therapeutic impact on this disease.

N-cadherin expression was associated with a loss or reduction in androgen receptor expression. N-cadherin-positive tumors expressed lower levels of androgen receptor than androgen-dependent control

tumors, and double-staining of LAPC9-CR tumors confirmed that androgen receptor was absent in a subset of N-cadherin-positive tumor cells. Forced N-cadherin expression resulted in androgen receptor loss proportional to the level of N-cadherin expression in LNCaP sublines. The mechanism by which N-cadherin reduces androgen receptor expression is not known. Additional studies will be required both to confirm this inverse correlation and to elucidate the pathway by which N-cadherin regulates androgen receptor. However, the major implication of our data is that N-cadherin may be a cause of androgen receptor-independent prostate cancer or may synergize with low-level androgen receptor expression. It will be crucial to determine whether N-cadherin can cause resistance to newer androgen receptor-targeted therapies.

The major findings of this paper are that N-cadherin can cause castration resistance and that therapeutic targeting of N-cadherin can delay CRPC progression. The mechanisms by which N-cadherin causes castration resistance, and by which N-cadherin-targeting antibodies inhibit it, are not known. However, N-cadherin activates gene encoding proteins previously implicated in castration resistance, such as IL-8, IL-6, TGF- $\beta$ , phosphoinositide 3-kinase and AKT, and bcl-2. For example, IL-8 is sufficient to cause castration resistance in androgen-dependent LNCaP and LAPC4 cells<sup>16</sup>. It has been shown that introduction of IL-8 leads to a decrease or loss in androgen receptor expression<sup>16</sup>, similar to what we saw with N-cadherin. N-cadherin-specific antibody 2A9 may act in part by reducing IL-8 secretion. Alternatively, the decrease in IL-8 could reflect the reduction in tumor volume caused by 2A9. One possible practical application of this observation would be to use IL-8 as a surrogate marker of antibody activity in future clinical trials. AKT has also been implicated in CRPC<sup>23</sup>. N-cadherin upregulated AKT activity, and exposure of PC-3 and LNCaP-C1 cells to 2A9 reduced this activity, even in PTEN-null cell lines. These data suggest that inhibition of N-cadherin-regulated AKT activation might be another mechanism by which 2A9 exerts its antitumor effect.

It is not clear why antibody 2A9 is superior to antibody 1H7 in some experiments. Although both antibodies could block invasion and metastasis, only 2A9 could reliably affect the growth of larger tumors or substantially delay progression to castration resistance. One possibility is that the epitope on the fourth extracellular domain recognized by 2A9 is essential for N-cadherin signaling, particularly in castration-resistant growth<sup>24</sup>. Alternatively, the differential activity could be related to differences in affinity or immune activation (antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity). Additional work will be required to understand the roles of the antibodies or the epitopes they recognize.

The finding that N-cadherin-targeted antibodies delay castration-resistant progression and inhibit growth, invasion and metastasis raises the possibility that these antibodies may be translatable to the clinic. Their toxicity is one question that needs to be addressed, as N-cadherin is expressed broadly in normal tissues such as peripheral nerve, heart and liver. Loss of N-cadherin can disrupt the intercalated disc structure in the heart, leading to ventricular tachycardia and sudden death in conditional-knockout mice<sup>25</sup>. Because 1H7 cross-reacts with mouse and human N-cadherin, we checked mice treated with 1H7 for signs of cardiac or other distress. Even at doses of 40 mg per kg body weight, we saw no evidence of toxicity, with no cases of sudden death, histologic heart abnormalities or changes in serum cardiac enzymes. These results suggest that therapeutic targeting of N-cadherin may be safe, although further preclinical and clinical testing will be required to confirm the safety of this approach.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

*Note: Supplementary information is available on the Nature Medicine website.*

## ACKNOWLEDGMENTS

This work was supported in parts by the US National Cancer Institute Prostate Cancer SPORE at the University of California–Los Angeles (P50CA092131-09 to R.E.R.), US Department of Defense Prostate Cancer Research grants (W81XWH-06-1-0324 to Z.A.W., W81XWH-09-1-0630 to R.E.R. and M.B.R., PC061456 to J.H.), Takeda Pharmaceuticals, the Jean Perkins Foundation and the American Cancer Society (RSG-07-092-01-TBE to J.H.). We also thank S. and L. Resnick, the Prostate Cancer Foundation and the Luskin Foundation for generous support and J. Said and N. Doan for immunohistochemical assessments. LNCaP-CL1 cells were provided by C.L. Tso (University of California–Los Angeles). Plasmid pΔVPR was provided by I. Chen (University of California–Los Angeles).

## AUTHOR CONTRIBUTIONS

H.T. and E.K. designed and conducted *in vitro* and *in vivo* studies. C.P.T. generated stable N-cadherin–knockdown reagents and prepared the manuscript. H.M. made the N-cadherin–overexpressing cell lines. J.Y. and R.W. performed gene and protein expression analyses. T.S. contributed to the *in vivo* N-cadherin–knockdown and antibody studies. F.L. and M.G. conducted immunohistochemical evaluation of prostate cancer specimens. J.H. contributed to immunohistochemical analyses of *in vivo* studies. R.L.V. provided clinical materials for the initial N-cadherin screening in metastases. J.A. and M.B.R. provided data on AKT activity. S.H. performed gene expression analysis for stem cell markers. Z.A.W. generated the monoclonal antibodies. R.E.R. conceived of the study and supervised the project. All authors discussed the results and commented on the manuscript at all stages.

## COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

Published online at <http://www.nature.com/naturemedicine/>.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>.

- Chen, C.D. *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**, 33–39 (2004).
- Suzuki, H., Ueda, T., Ichikawa, T. & Ito, H. Androgen receptor involvement in the progression of prostate cancer. *Endocr. Relat. Cancer* **10**, 209–216 (2003).
- Mellado, B., Codony, J., Ribal, M.J., Visa, L. & Gascon, P. Molecular biology of androgen-independent prostate cancer: the role of the androgen receptor pathway. *Clin. Transl. Oncol.* **11**, 5–10 (2009).
- Harris, W.P., Mostaghel, E.A., Nelson, P.S. & Montgomery, B. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. *Nat. Clin. Pract. Urol.* **6**, 76–85 (2009).

- Roudier, M.P. *et al.* Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone. *Hum. Pathol.* **34**, 646–653 (2003).
- Shah, R.B. *et al.* Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **64**, 9209–9216 (2004).
- Lassi, K. & Dawson, N.A. Emerging therapies in castrate-resistant prostate cancer. *Curr. Opin. Oncol.* **21**, 260–265 (2009).
- Attard, G., Reid, A.H., Olmos, D. & de Bono, J.S. Antitumor activity with CYP17 blockade indicates that castration-resistant prostate cancer frequently remains hormone driven. *Cancer Res.* **69**, 4937–4940 (2009).
- Rizzi, F. & Bettuzzi, S. Targeting clusterin in prostate cancer. *J. Physiol. Pharmacol.* **59**Suppl 9, 265–274 (2008).
- Gleave, M., Miyake, H. & Chi, K. Beyond simple castration: targeting the molecular basis of treatment resistance in advanced prostate cancer. *Cancer Chemother. Pharmacol.* **56**Suppl 1, 47–57 (2005).
- Sharifi, N., Kawasaki, B.T., Hurt, E.M. & Farrar, W.L. Stem cells in prostate cancer: resolving the castrate-resistant conundrum and implications for hormonal therapy. *Cancer Biol. Ther.* **5**, 901–906 (2006).
- Isaacs, J.T. The biology of hormone refractory prostate cancer. Why does it develop? *Urol. Clin. North Am.* **26**, 263–273 (1999).
- Gu, Z. *et al.* Reg IV: a promising marker of hormone refractory metastatic prostate cancer. *Clin. Cancer Res.* **11**, 2237–2243 (2005).
- Tso, C.L. *et al.* Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J.* **6**, 220–233 (2000).
- Tran, N.L., Adams, D.G., Vaillancourt, R.R. & Heimark, R.L. Signal transduction from N-cadherin increases Bcl-2. Regulation of the phosphatidylinositol 3-kinase/Akt pathway by homophilic adhesion and actin cytoskeletal organization. *J. Biol. Chem.* **277**, 32905–32914 (2002).
- Araki, S. *et al.* Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. *Cancer Res.* **67**, 6854–6862 (2007).
- Domingo-Domenech, J. *et al.* Interleukin 6, a nuclear factor-κB target, predicts resistance to docetaxel in hormone-independent prostate cancer and nuclear factor-κB inhibition by PS-1145 enhances docetaxel antitumor activity. *Clin. Cancer Res.* **12**, 5578–5586 (2006).
- Tomita, K. *et al.* Cadherin switching in human prostate cancer progression. *Cancer Res.* **60**, 3650–3654 (2000).
- Jaggi, M. *et al.* N-cadherin switching occurs in high Gleason grade prostate cancer. *Prostate* **66**, 193–199 (2006).
- Gravdal, K., Halvorsen, O.J., Haukaas, S.A. & Akslen, L.A. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. *Clin. Cancer Res.* **13**, 7003–7011 (2007).
- Mani, S.A. *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704–715 (2008).
- Mason, M.J., Fan, G., Plath, K., Zhou, Q. & Horvath, S. Signed weighted gene co-expression network analysis of transcriptional regulation in murine embryonic stem cells. *BMC Genomics* **10**, 327 (2009).
- Majumder, P.K. & Sellers, W.R. Akt-regulated pathways in prostate cancer. *Oncogene* **24**, 7465–7474 (2005).
- Kim, J.B. *et al.* N-Cadherin extracellular repeat 4 mediates epithelial to mesenchymal transition and increased motility. *J. Cell Biol.* **151**, 1193–1206 (2000).
- Li, J. *et al.* Cardiac-specific loss of N-cadherin leads to alteration in connexins with conduction slowing and arrhythmogenesis. *Circ. Res.* **97**, 474–481 (2005).

## ONLINE METHODS

**Cell culture.** LNCaP-FGC, MDA-PCa-2b and PC-3 cells were from the American Type Culture Collection and cultured as specified. LNCaP-CL1 cells<sup>14</sup> were provided by C.L. Tso and maintained in phenol-red-free RPMI-1640 with 10% charcoal stripped serum (DCC-FBS). LAPC4 and LAPC9 xenografts were passaged in male severe combined immunodeficient (SCID) mice<sup>26</sup> (Taconic).

**Antibodies.** Western blot analysis was performed as previously described<sup>27</sup> with antibodies specific for N-cadherin (3B9 from Invitrogen, clone 32 from BD Transduction Laboratories), E-cadherin (Zymed Laboratories), vimentin (Thermo Scientific), androgen receptor, glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology), AKT and phospho-AKT (Cell Signaling Technology). Immunohistochemistry was performed as previously described<sup>28</sup>, with antibodies specific for CD31 (Santa Cruz Biotechnology), vimentin, Ki-67 (DakoCytomation) and caspase-3 (Cell Signaling Technology). Flow cytometry was performed with N-cadherin-specific antibody GC-4 (Sigma).

**Establishment of N-cadherin-overexpressing and N-cadherin-knockdown cells.** Full-length N-cadherin cDNA was subcloned into the lentiviral vector CSCG (Addgene) to make CSCG-N-cadherin. The lentiviral stock was produced in 293T cells by transfecting 6.25 µg CSCG-N-cadherin, 2.5 µg envelope plasmid VSVG and 6.25 µg packaging plasmid pΔVPR (provided by I. Chen). LNCaP-FGC, MDA-PCa-2b and LAPC4 cells were transduced with either CSCG-N-cadherin or CSCG-GFP lentiviruses and sorted for positive cells. LNCaP-C1, LNCaP-C2 and LNCaP-C3 were derived through limiting dilution and screening individual clones by RT-PCR for varying levels of N-cadherin and androgen receptor expression. For stable knockdown, shRNA against N-cadherin was subcloned into the GFP-positive lentiviral vector FG-12 (Addgene) to make FG12-shNcad. Scrambled shRNA was also used to make control vector. Lentiviruses were produced as described above and used to transduce PC3 and CL1 cells. One week after transduction, FG12-shNcad-transduced cells were labeled with N-cadherin-specific antibody and sorted by flow cytometry, gating for a GFP-positive, N-cadherin<sup>low</sup> population. The cell lines with control vector were not sorted but were confirmed to be >50% GFP positive. After the sort, cells were immediately implanted in castrated mice as described below (*in vivo* assays).

**Purification and characterization of N-cadherin-specific monoclonal antibodies.** The 1H7 (IgG1-κ) and 2A9 (IgG2a-κ) N-cadherin-specific hybridomas were raised against His-tagged N-cadherin proteins representing the first three and fourth extracellular domains as previously described<sup>26</sup> and screened by ELISA<sup>13</sup> and FACS. Hybridomas were cultured in HL-1 medium (Lonza) in Integra CL 1000 flasks following the manufacturer's instructions (IBS Integra Biosciences). 1H7 and 2A9 monoclonal antibodies were purified by protein-G affinity chromatography (GE), and BIAcore 3000 (Precision Antibody Service) analysis was done with recombinant His-tagged N-cadherin as antigen.

**In vitro assays.** Cell proliferation was measured with the CCK-8 kit (Dojindo). For attachment assays, collected cells were pretreated with 1× PBS or 80 µg ml<sup>-1</sup>

2A9 antibody at 37 °C for 2 h, plated in fibronectin-coated 96-well plates without or with 2A9 for 15 min, and washed twice with 1× PBS. Attached cells were quantified by crystal violet staining. Invasion assays were performed in 24-well Matrigel invasion chambers (BD Biosciences) as previously described<sup>26</sup>, in the presence of PBS control or 80 µg ml<sup>-1</sup> 2A9 for 48 h. For knockdown experiments, cells were first transfected with commercial N-cadherin and nontargeting (control) siRNA pools, and 24 h later plated into invasion chambers, followed by quantification of invasion 48 h later. Two different pools of N-cadherin siRNA (Santa Cruz Biotechnology and Dharmacon) were used to verify the results in both cell lines.

**Interleukin-8 assay.** Conditioned media or mouse sera (50 µl) were assayed for IL-8 with the Human IL-8 (CXCL8) ELISA Kit (R&D Systems). Mouse blood samples were obtained retro-orbitally, and sera were separated by centrifugation.

**In vivo studies.** All *in vivo* experiments were performed according to approved protocols from the Animal Research Committee at the University of California–Los Angeles. PC3 and N-cadherin-expressing LNCaP cells (1 × 10<sup>6</sup> cells) in 50% Cultrex (Trevigen) were implanted subcutaneously in 6- to 8-week-old castrated male nude (Charles River) and SCID mice, respectively. LAPC9-CR xenograft tumors were collected from castrated male SCID mice and processed to single-cell suspensions as previously described<sup>26</sup>. We injected 1 × 10<sup>6</sup> cells subcutaneously into 6- to 8-week-old castrated male SCID mice. N-cadherin-specific antibody (500 µl) at 10 or 20 mg per kg body weight, or 1× PBS control was injected intraperitoneally twice weekly, when the tumors were palpable, 100 mm<sup>3</sup> in size or 200 mm<sup>3</sup> in size. Tumors were measured with calipers, and tumor volume was calculated as follows: (larger diameter) × (smaller diameter) × (third diameter, or width). To show the specificity of the N-cadherin-specific antibody, we did an initial experiment with N-cadherin-negative LAPC9-AD and N-cadherin-positive LAPC9-CR tumors, which showed antibody efficacy only in the positive tumors (data not shown).

For progression to castration-resistant studies, LAPC9-AD xenograft tumors were collected from intact male SCID mice, and 1 × 10<sup>6</sup> cells were injected subcutaneously into either intact or castrated 6- to 8-week-old male SCID mice pretreated with either PBS control or 10 mg kg<sup>-1</sup> body weight N-cadherin-specific antibody. Thereafter, treatments were performed twice a week, and tumor volumes were monitored as described above.

**Statistical analyses.** Data are shown as means ± s.e.m. Where indicated, *P* values were determined by unpaired Student's *t* test. *P* ≤ 0.05 was considered significant.

26. Klein, K.A. *et al.* Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat. Med.* **3**, 402–408 (1997).

27. Hara, T., Miyazaki, H., Lee, A., Tran, C.P. & Reiter, R.E. Androgen receptor and invasion in prostate cancer. *Cancer Res.* **68**, 1128–1135 (2008).

28. Gu, Z. *et al.* Prostate stem cell antigen (PSCA) expression increases with high Gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene* **19**, 1288–1296 (2000).