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Award Number: W81WXH-06-1-0324

TITLE: DEVELOPMENT OF ANTIBODIES AGAINST NOVEL CELL SURFACE PROTEINS IN HORMONE REFRACTORY PROSTATE CANCER

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REPORT DATE: July 2011

TYPE OF REPORT: Final Addendum

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2011	Final Addendum	1 July 2010 – 30 June 2011

4. TITLE AND SUBTITLE

DEVELOPMENT OF ANTIBODIES AGAINST NOVEL CELL SURFACE PROTEINS IN HORMONE REFRACTORY PROSTATE CANCER

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81WXH-06-1-0324

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California
Los Angeles, CA 90095

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

N-cadherin is a cell surface marker that is overexpressed in hormone refractory prostate cancer and targeting this protein either diagnostically or therapeutically may have clinical utility. We developed monoclonal antibodies against several ectodomains of N-cadherin that 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 tumor regression. In addition, N-cadherin antibodies markedly delayed the time to emergence of castration resistance, markedly affected tumor histology, angiogenesis, and reduced both AKT activity and serum IL-8 secretion. These data demonstrate that N-cadherin is a significant cause of both prostate cancer metastasis and castration resistance and therapeutic targeting of this factor with monoclonal antibodies may have significant clinical benefit. For the final year of the grant, there has been an increased attempt to identify the mechanism of action of these antibodies and their effects on downstream signaling pathways.

15. SUBJECT TERMS
N-cadherin, preclinical mouse model, antibody therapy, antitumor activity, hormone refractory prostate cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	21	USAMRMC

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INTRODUCTION

We have previously identified N-cadherin as a target that contributes to and is required for both metastasis and castration resistance in prostate cancer. Over the course of this grant, our lab developed monoclonal antibodies against the extracellular domains 1-3 (1H7) and extracellular domain 4 (2A9) of N-cadherin which were used both *in vitro* and *in vivo* to treat N-cadherin positive xenografts. These antibodies are able to block invasion and metastasis, inhibit growth, and delay castration resistance *in vivo*. Therapeutic targeting of N-cadherin with monoclonal antibodies, alone or in combination with other drugs targeting those markers potentially involved in downstream pathways may have significant clinical benefit.

PROGRESS REPORT

Specific Aim 1. To Validate N-Cadherin and Ly6E as Targets for therapy

Previous annual reports have detailed the process whereby N-cadherin was selected and then validated as an appropriate target for further investigation. We performed a series of experiments (detailed in previous annual reports, years 1-3) that indicated that N-cadherin was significantly overexpressed and upregulated in prostate cancer tissue when compared to normal controls. In addition, as previously reported, we constructed lentiviral vectors of N-cadherin which were then used to demonstrate that N-cadherin was a stimulator of cellular invasion. We used boyden chamber assays to confirm that N-cadherin was responsible for aggressive cellular invasion in several *in vitro* models.

Since the last annual report, we have performed several new experiments which add insight to N-cadherin function in prostate cancer. In these experiments, N-cadherin positive prostate cancer cell lines PC3, LNCaP-C1, C2, C3, (where C1>C2>C3 for N-cadherin expression) CL1 and N-cadherin negative cell line LNCaP-FGC were used. LNCaP-C1, C2 and C3 were N-cadherin overexpression lines derived from lentiviral infection of LNCaP-FGC. LNCaP-CL1 expressing N-cadherin endogeneously was generated from LNCaP-FGC using androgen starved culture media. In these experiments, these cells were treated with either monoclonal antibodies raised against the extracellular domain of N-cadherin or with siRNA knockdown. As demonstrated in **figure 1**, we were able to demonstrate that stable silencing of N-cadherin impaired the ability of both cell lines (PC3 and LNCaP-CL1) to form tumors in castrated mice.

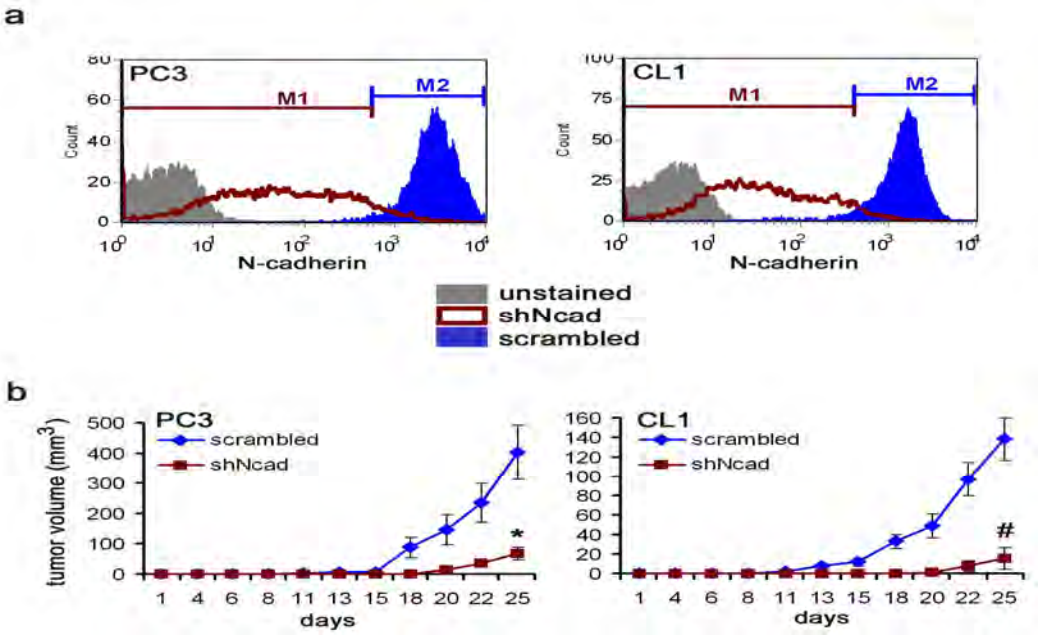

Figure. 1. Endogenous N-cadherin shRNA knockdown (shNcad) suppressed castrate resistant growth of prostate cancer xenografts PC3 and CL1. Both cell lines were transduced with shNcad or control (scramble shRNA) GFP-lentivector, sorted for the GFP positive population, followed by subcutaneous implantation in castrated nude mice. **(a)** FACS analyses of transduced PC3 and CL1 cells 2 weeks after sorting. Note that N-cadherin positivity decreased markedly, as depicted by the peak shift from M2 to M1. **(b)** *In vivo* castrate resistant tumor growth of transduced PC3 and CL1. Significant tumor inhibition was observed in the shNcad tumor group for both xenograft models. *P = 0.005, #P = 0.001.

Specific Aim 2. Antibody Development:

As we detailed in previous reports, we generated stable recombinant N-cadherin in 2 separate vector systems and then began to generate several monoclonal antibodies. Previous reports have detailed both the methodology used to generate the antibodies (hybridomas, phage display library) and characterization of the antibodies (isotyping). As also detailed in previous annual reports, despite multiple attempts, the gene Ly6E was never sufficiently stable in a vector system so no successful antibodies were produced. This protein is heavily glycosylated and this may have contributed to this challenge.

As first reported in past annual reports, antibodies were developed against several ectodomains of N-cadherin in order to select which parts of the protein were more amenable to antibody targeting. Because of the specific importance the 4th extracellular domain plays in motility and invasion, it was of particular interest. In fact, when compared to the other antibody generated (1H7), the anti-EC4 antibody (2A9) had a greater effect on *in vivo* growth inhibition and as detailed in this report was pursued more aggressively in the *in vivo* experiments.

Specific Aim 3. Preclinical Testing and mechanism of action of anti-N-cadherin antibodies:

As also detailed in previous annual reports (years 2-3), anti-N-cadherin antibodies showed dramatic growth inhibition in several prostate cancer xenograft models and seemed to decrease rates of metastasis as well. Furthermore, while the exact mechanism of action of these antibodies was not clear, the NF KappaB, FGFR and angiogenesis pathways were implicated in previous experiments. The next series of experiments were done to identify the mechanism of action of these antibodies with an emphasis on downstream signaling.

To gain further insight into the mechanism of N-cadherin activity in prostate cancer and the effects of the antibodies, we compared expression profiles of N-cadherin transduced cell lines and controls. While previous studies have associated N-cadherin with phosphoinositide 3-kinase–AKT pathway activation, we also selected many genes associated with castrate resistance. As predicted, N-cadherin transduced cells showed characteristic changes of an EMT with decreased E-cadherin, TGF-B1, VEGF and reduced IL-6 and IL-8 (**Figure 2a**). N-cadherin silencing also reduced AKT phosphorylation, whereas N-cadherin overexpression correlated with increased AKT activity (**Figure. 2 b**,**c**). These results indicate that N-cadherin is sufficient to cause EMT and regulates the expression of multiple genes implicated in castration resistance. Therefore, 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 expression and AKT kinase activity over a 4- to 24-h time period (**Fig. 2d**). ELISA of cell culture media after 2A9 treatment showed a >50% reduction in IL-8 secretion (**Fig. 2e**). 2A9 treatment also led to progressive declines in serum IL-8 that correlated with antibody dose and tumor regression (**Fig. 2f**). 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. Furthermore, we hypothesized that the importance of IL-8 could serve as a potential biomarker of N-cadherin and N-cadherin–targeted therapy.

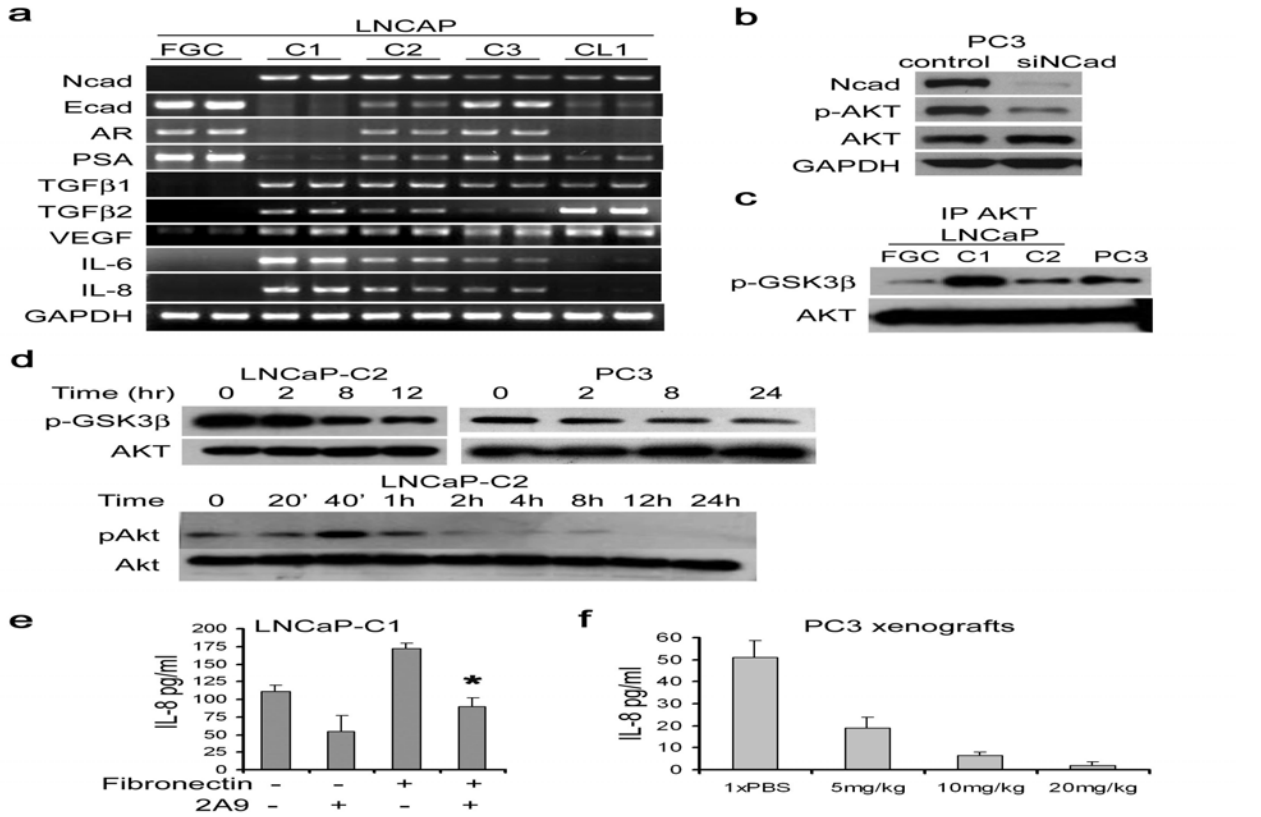

Figure 2. (a) RT-PCR analyses of gene expression in LNCaP cells without (FGC) or with high (C1, C2 and CL-AI) and low (C3) levels of N-cadherin. (b) Western blot of PC3 cells upon N-cadherin siRNA silencing (siN-cad). (c) AKT kinase activity in N-cadherin–overexpressing cell lines, measured by *in vitro* kinase assay. (d) Changes in AKT kinase activity and phospho-AKT level in *in vitro* time-course treatment with 2A9 at 80 μg ml−1 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 μg ml−1 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 ± s.e.m.

In addition, while silencing of N-cadherin in PC3 cells decreased Il-6, IL-8 and others, not surprisingly there were no changes in E-cadherin or AR expression. This would suggest as implied in **figure 3** that a more prolonged knockdown might be required for complete reversal of the EMT.

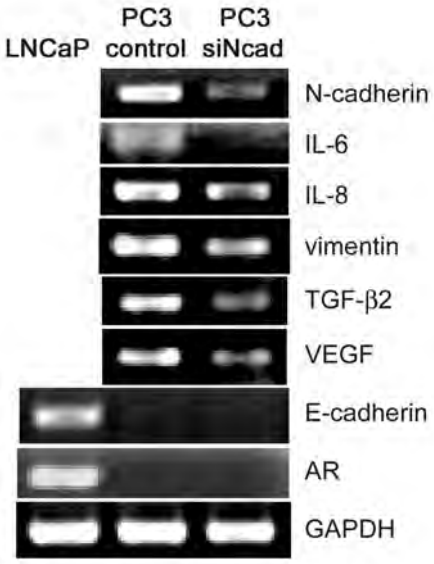

Figure 3. Gene expression was determined by semi-quantitative RT-PCR; LNCaP cell was included as positive control for E-cadherin and AR.

Our interest in Il-6 and IL-8 was partly enhanced by the fact that these two cytokines are themselves target genes of NF KappaB. As indicated below, in **figure 4**, there is upregulation of both IL-6 and IL-8 that correlated with N-cadherin overexpression in LNCap N-cadherin transduced cell lines.

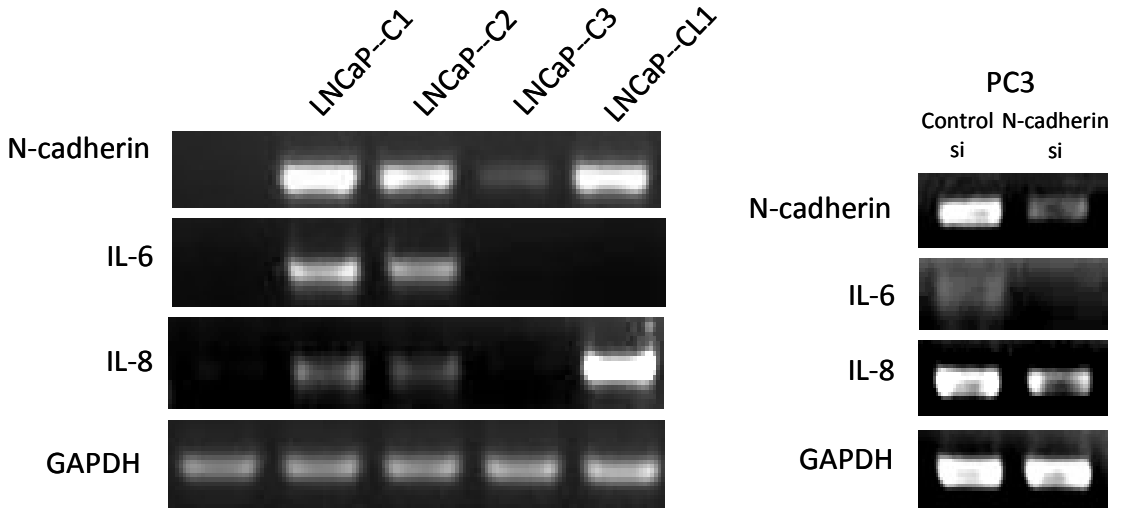

Figure 4. Semi quantitive RT-PCR shows that IL-6 and IL-8 mRNA expression correlates with N-cadherin expression in LNCaP N-cadherin overexpression lines. In PC3 cell line, expression of IL-6 and IL-8 is downregulated when N-cadherin is knocked down by siRNA.

When the relationship between the antibody and its effects was tested *in vivo*, we were able to demonstrate that treatment with the N-cadherin antibody showed a significant decrease in IL-8 in blood serum. Interestingly, while the effects on Il-8 were seen with both antibodies, it was more pronounced in the 2A9 treated mice consistent with previous *in vitro* experiments.

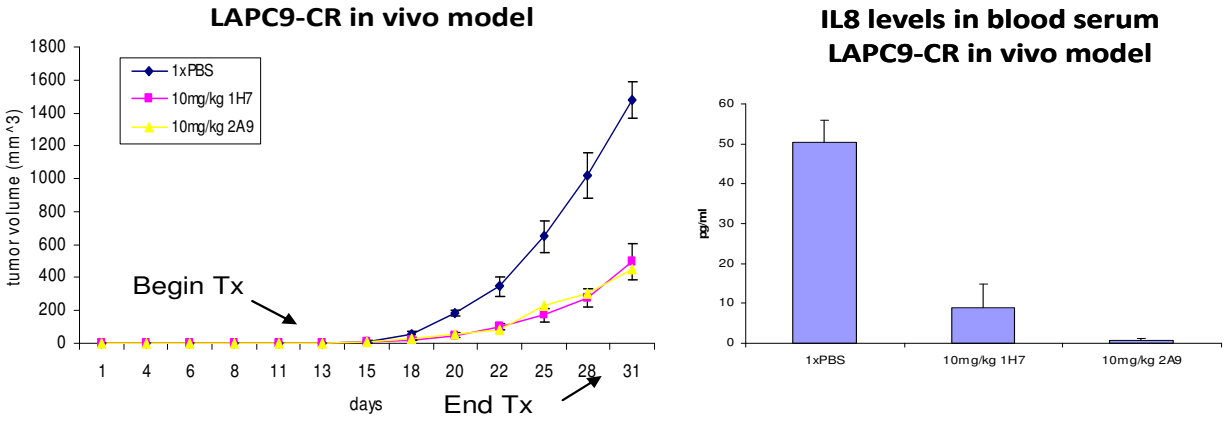

Figure 5. Mice were injected with LAPC9-CR subcutaneously over the flank and treatment began when tumors were palpable. Treatment with either antibody compared to control slowed tumor growth significantly in several N-cadherin positive cell lines *in vivo*. Blood serum showed decrease in IL-8 in antibody treated groups. Antibody treatment in N-cadherin negative cell lines, such as LAPC9AD, did not affect tumor growth *in vivo* (data not shown).

Another hypothesized mechanism of action of our antibody related to the fibroblast growth factor receptor (FGFR) pathway which has already been implicated in cadherin biology in breast cancer cells.
As seen in **figure 6**, FGF2 mRNA expression correlated with N-cadherin expression. Expression of FGF2 was detected in LNCaP-C1, an exogenously N-cadherin expressing LNCaP cell line, as well as the N-cadherin positive PC3 cell line, while the N-cadherin negative LNCaP line did not. siRNA knockdown of Ncadherin resulted in downregulation of FGF2 in PC3 cells. FGFR1 expression was independent of N-cadherin expression. Finally, antibody treatment with 2A9 treatment of PC3 cells affected AKT phosphorylation by FGF2. We have previously shown that N-cadherin expression correlates with AKT activity and it is known that FGF2 increases AKT phosphorylation of ser473 in PC3 cells. We also used boyden chamber invasion assays **(figure 7)** to show that the 2A9 antibody block increase in invasion by FGF2 in PC3 cell line, suggesting specificity of this antibody that was improved over the 1H7 antibody. Not surprisingly**,** n-cadherin siRNA knockdown affected invasion in PC3 cells in the presence of FGF2.

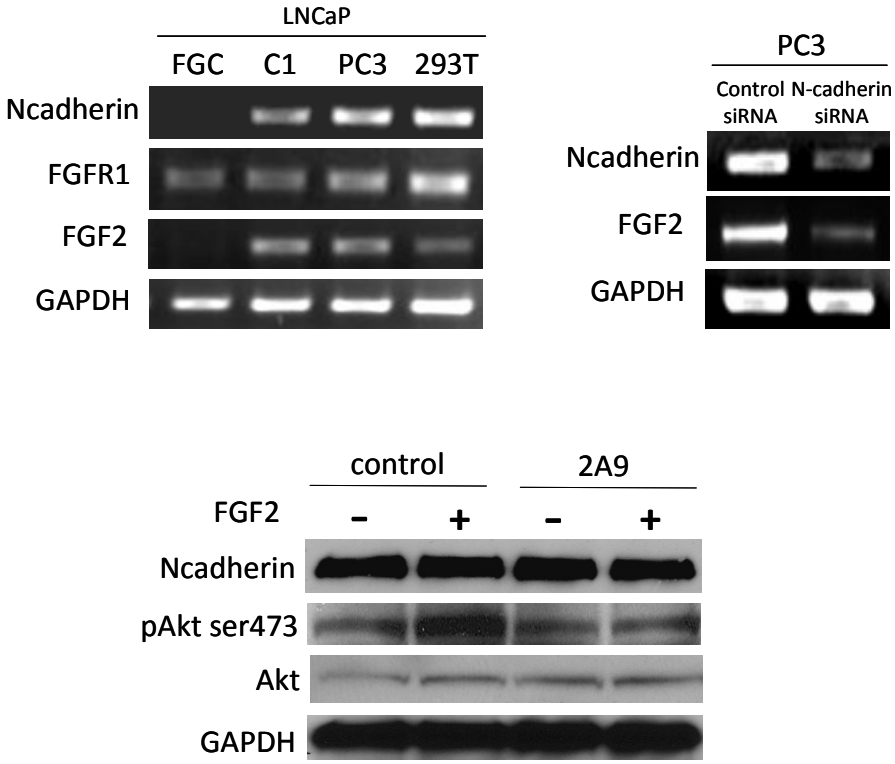

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Figure 6. Semi quantitive RT-PCR shows FGF2 mRNA expression correlates with N-cadherin expression in N-cadherin overexpression lines. In PC3 cell line, expression of FGF2 is downregulated when N-cadherin is knocked down by siRNA. Treatment of PC3 cells with 80ug/ml 2A9 antibody affects the increase in phosphorylation by FGF2.

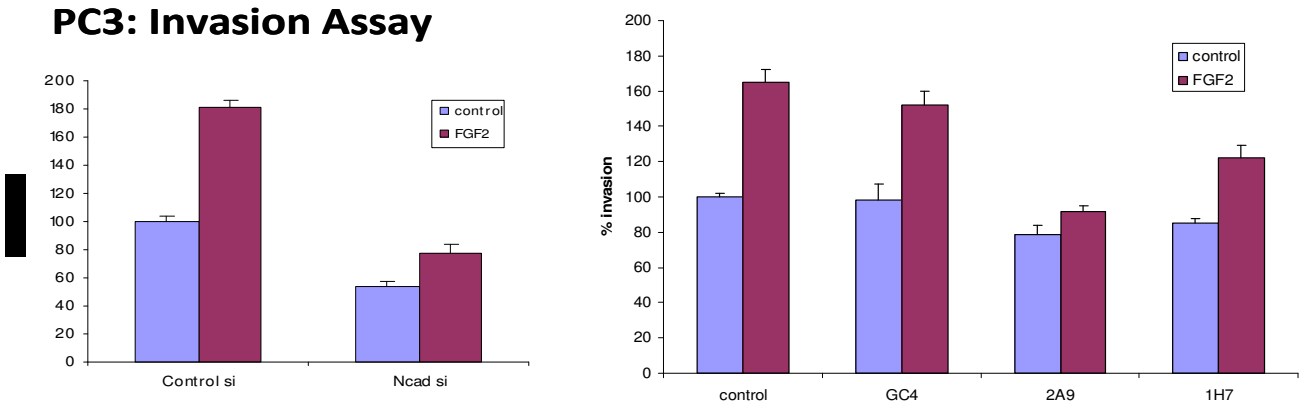
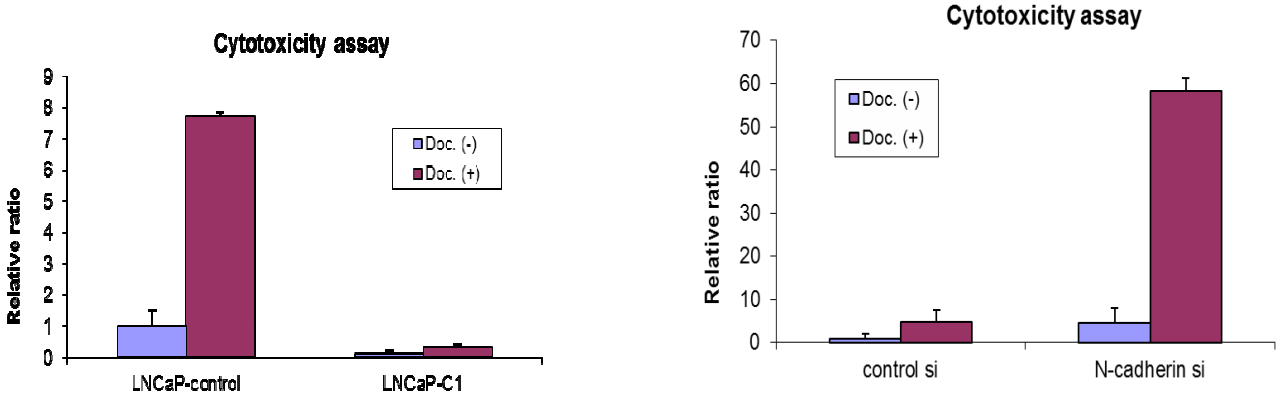


Figure 7. Treatment of N-cadherin positive PC3 cell line with 10ng/ml FGF2 increase invasion in vitro. 2A9 antibody treated PC3 cells invaded less in comparison to 1H7 and the commercial GC4 (SIGMA) antibody treated cells in the presence of added FGF2.

Pre-clinical testing-

In clinical oncology, most successful antibodies are combined with either chemotherapy or other agents. The next series of experiments set the stage for the most rational and effective combination strategies. Docetaxel is the standard of chemotherapeutic agent given to men with castration-resistant prostate cancer (CRPC). However, many patients do not respond or develop resistance to this treatment. In these studies we sought to determine the role of N-cadherin expression in docetaxel resistant models and whether treatment with the N-cadherin monoclonal antibodies restored chemosensitivity. We initially determined that cells that overexpressed N-cadherin were in fact less sensitive to docetaxel than N-cadherin negative cells. This effect was reversed when cells were exposed to the N-cadherin siRNA knockdown.

Figure 8. Cytotoxicity assay (LDH detection kit) was performed on N-cadherin overexpressing cell line LNCaP-C1 in the presence of 2.5nM Docetaxel and compared to the control. The results show less cytotoxicity with docetaxel in LNCaP-C1, in comparison to the N-cadherin negative LNCaP-control cell line. N-cadherin knockdown increases cytotoxicity in PC3 cells in the presence of docetaxel. N-cadherin in PC3 cell line was knocked down with siRNA then treated with 2.5nM docetaxel. Cytotoxicity assay shows N-cadherin knockdown resulted in a 12-fold increase in cytotoxicity when treated with docetaxel when compared to control.



Interestingly, the combination treatment of docetaxel and 2A9 N-cadherin monoclonal antibody increased cytotoxicity in N-cadherin positive PC3 cell line in vitro**.** This would suggest that more pronounced cytoxic effects can be seen with the combination of the chemotherapy and the N-cadherin antibody.

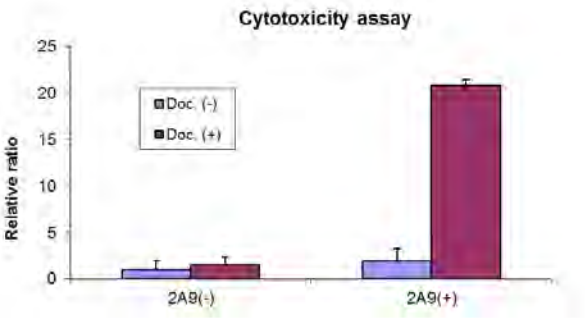


Figure 9. PC3 cells were either treated along or in combination of 2.5nM Docetaxel and 80ug/ml 2A9 N-cadherin antibody. Combination treatment with Docetaxel and 2A9 antibody showed significant increased in cytotoxicity. Similar results were observed in LNCaP-CL1 cell line.

In order to expand these *in vitro* observations to an *in vivo* model, we described in the 2010 annual report, a series of dose escalation experiments for the N-cadherin antibody with docetaxel in the PC3 xenograft model. As can be seen in **figure 10**, we have now successfully demonstrated that the 2A9 monoclonal antibody 2A9 has additive effect to docetaxel in this model and that the combination is more efficacious that either docetaxel or the antibody alone.

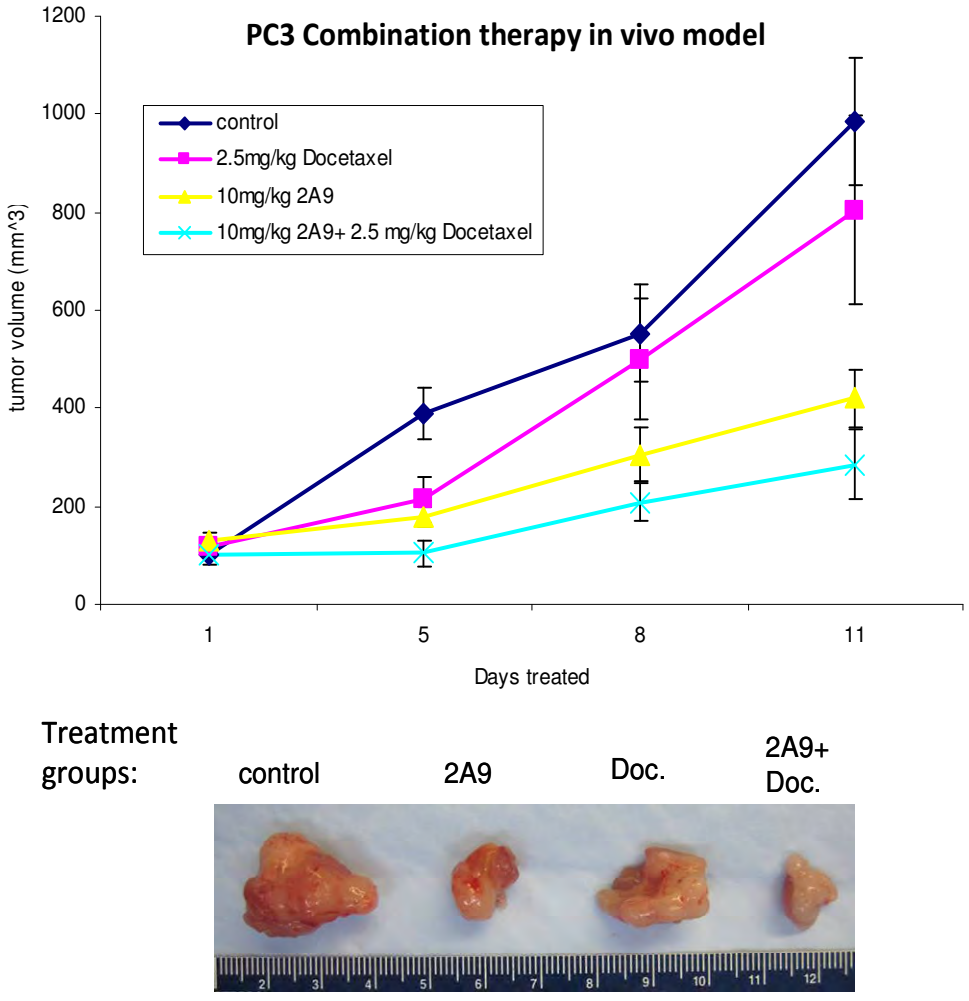


Figure 10. PC3 cells were injected subcutaneously over the flank and treated when the tumor size reached 100mm3, treatments were started. Combination of Docetaxel and N-cadherin monoclonal antibody 2A9 has greater efficacy than either 2A9 or Docetaxel alone. Upper panel: Tumor growth curve, Lower panel: Photograph of tumors (average size). Mice were treated with 10mg/kg 2A9 twice a week and/or 2.5mg/kg Docetaxel once a week.

KEY RESEARCH ACCOMPLISHMENTS

- N-cadherin specific antibodies inhibited tumor growth, decreased invasion, lowered proliferation, and lowered angiogenesis. The data demonstrates that the antibodies suppress the growth of large established tumors and that higher doses can cause tumor regression. The antibodies also prevented androgen independence in several models.
- The above 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 extracellular domain of N-cadherin. Therapeutic targeting of N-cadherin with monoclonal antibodies, alone or in combination with other drugs targeting those potentially involved in downstream pathways may have significant clinical benefit.
- These studies also suggest that N-cadherin is involved in the development of chemoresistance in prostate cancers. Therapeutic targeting of N-cadherin with monoclonal antibodies, in combination with therapeutic agents such as docetaxel may have significant clinical benefit. Future experiments involve expanding on the combination therapy of docetaxel and N-cadherin antibody and with other combinations as well (abiraterone, AKT inhibitors, PI3Kinase inhibitors etc..).

REPORTABLE OUTCOMES

Tanaka H, Kono E, Tran CP, Miyazaki H, Yamashiro J, Shimomura T, Fazli L, Wada R, Huang J, Vessella RL, An J, Horvath S, Gleave M, Rettig MB, Wainberg ZA, Reiter RE.
Monoclonal antibody targetting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance. Nat Med. 2010 Dec; 16(12):1414-20.

N-cadherin monoclonal antibody as a therapeutic agent against chemoresistance in prostate cancer
Tatsuya Shimomura, Evelyn Kono, Chau P. Tran, Joyce Yamashiro, Hiroshi Tanaka, Michael McGuire, Zev Wainberg, and Robert E. Reiter
Presented at AACR 102nd Annual Meeting, Orlando, Florida, April 2-6, 2011

Monoclonal antibody against the 4th extracellular domain of N-cadherin affects FGF2 mediated pathways in prostate cancer
Evelyn Kono, Chau P. Tran, Joyce Yamashiro, Tatsuya Shimomura, Hiroshi Tanaka, Michael McGuire, Zev Wainberg, and Robert E. Reiter
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CONCLUSION

Initial experiments indicated that N-cadherin was a great target for antibody development. Our efforts have been spent developing and optimizing our anti N-cadherin antibodies which are now very well characterized and are being continually developed.

During this period of the grant, I have been attending career development activities including classes and lectures including: NIH Principles of Clinical Pharmacology, General Clinical Annual Research Course and NIH Grant Preparation Courses. In addition to these classes, I have also participated in many Prostate Cancer SPORE-related activities, including lectures, symposia and journal clubs and have since been promoted to Assistant Professor of Medicine at UCLA. Furthermore, the preliminary data obtained in this award was instrumental in helping me obtain a Prostate Cancer Spore Career Development Award. I have gratefully acknowledged the contribution of this award in all abstracts and publications.

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nature medicine

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

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

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[1–4]. 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[5,6]. 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[7,8]. 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[9,10]. 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[11,12]. 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

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

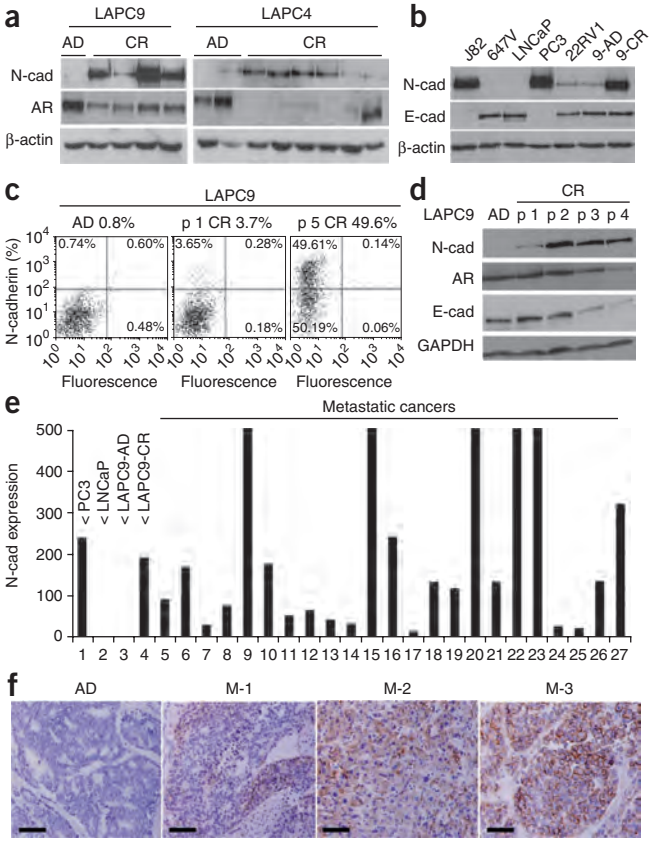

Figure 1 N-cadherin is upregulated in castration resistant prostate cancer. (**a**) N-cadherin and androgen receptor expression in multiple independently derived paired AD and CR LAPC4 and LAPC9 xenografts. (**b**) Protein expression of N-cadherin and E-cadherin in prostate cancer cells lines (LNCaP, 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 μm.

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[13]. N-cadherin expression was highly elevated in LAPC9-CR xenografts[13], 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[14] 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-naive 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-naive 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

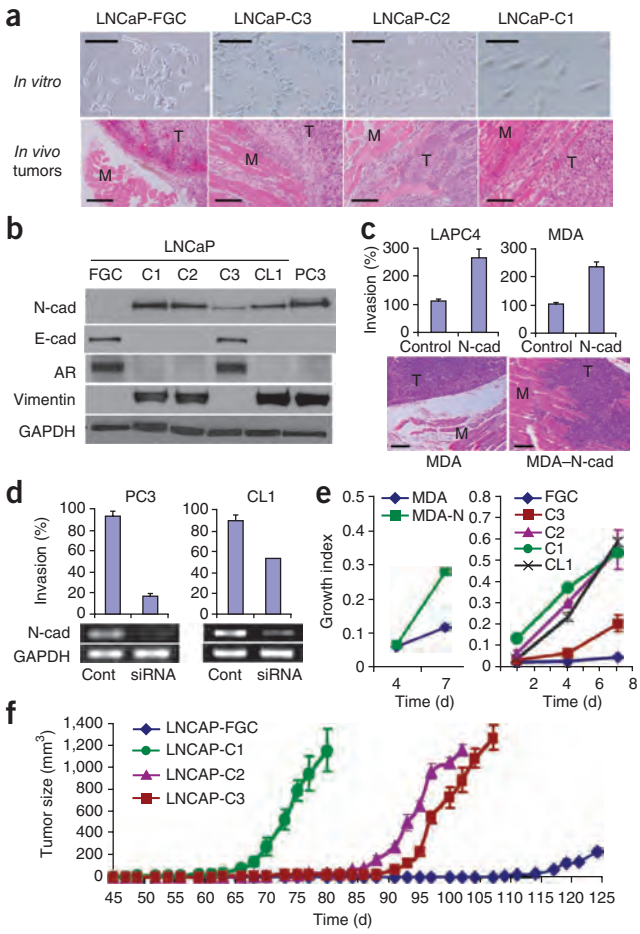

Figure 2 N-cadherin causes invasion, migration and EMT of multiple prostate cancer cell lines. (**a**) Top, *in vitro* morphologic changes in LNCaP sublines that overexpress increasing amounts of N-cadherin (LNCaP-C3 < LNCaP-C2 < LNCaP-C1) compared to control cell line LNCaP-FGC (control). Scale bar, 50 μm. 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 μm. (**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 μm. M, muscle; T, tumor. (**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 ± 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.

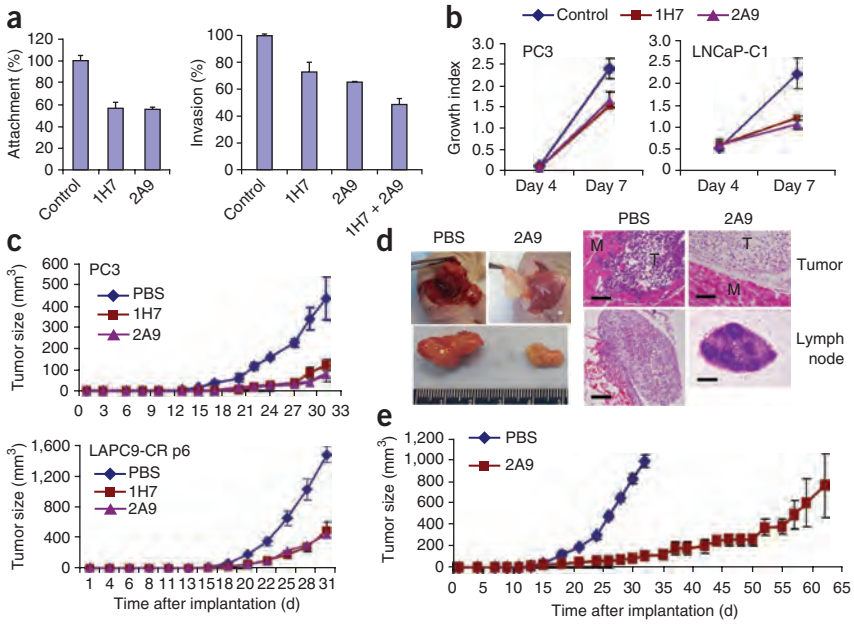

Figure 3 Antibodies against N-cadherin decrease invasion and tumor growth. (**a**) Attachment ($P = 0.004$) and invasion ($P = 0.05$) assays of PC3 cells upon treatment without or with 1H7 or 2A9 (80 μg ml^{-1}). (**b**) Decrease in cell growth measured by proliferation assays of PC3 ($P = 0.014$) and LNCaP-C1 ($P = 0.01$) cells, upon treatment without or with 80 μg ml^{-1} 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 μm) and metastases to axillary lymph nodes (scale bar, 500 μm). (**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 ± 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

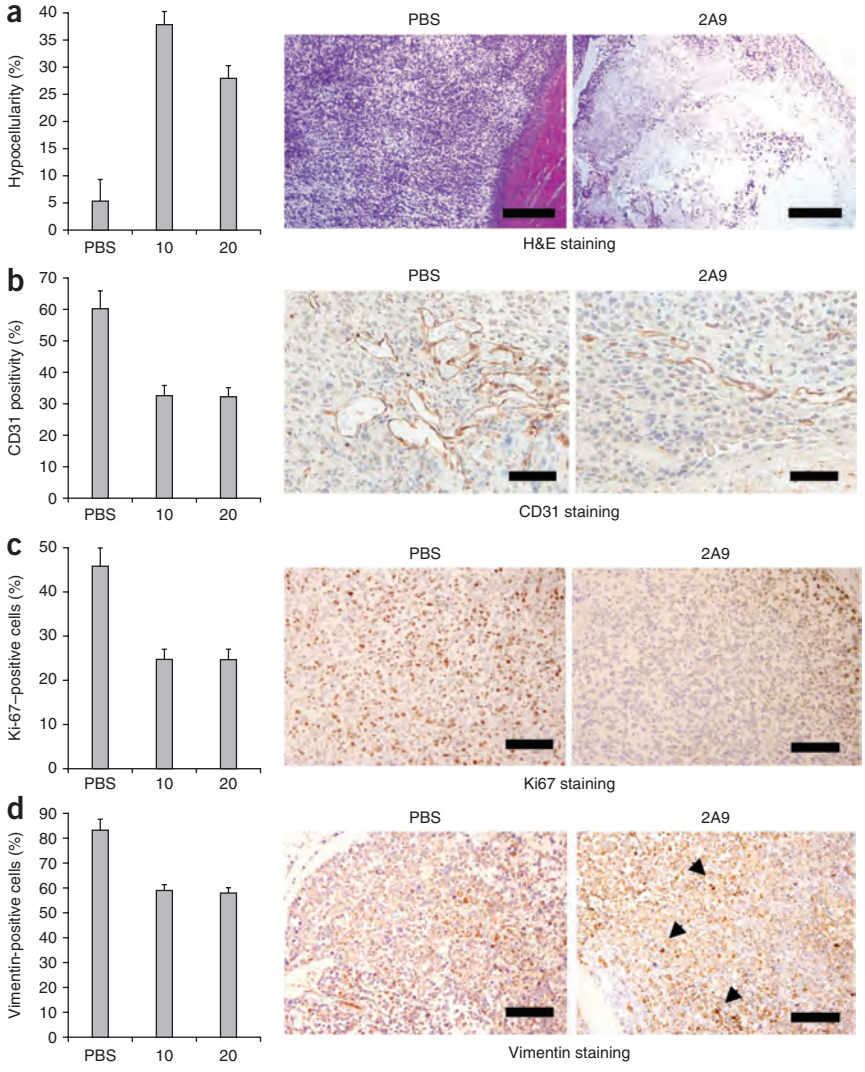

Figure 4 Histological and immunohistochemical assessments of PC3 tumors treated without or with 2A9 at 10 or 20 mg per kg body weight. (**a**–**d**) Quantification of changes between untreated and treated tumors in the following parameters: hypocellular regions ($P = 0.004$) by H&E staining (scale bar,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

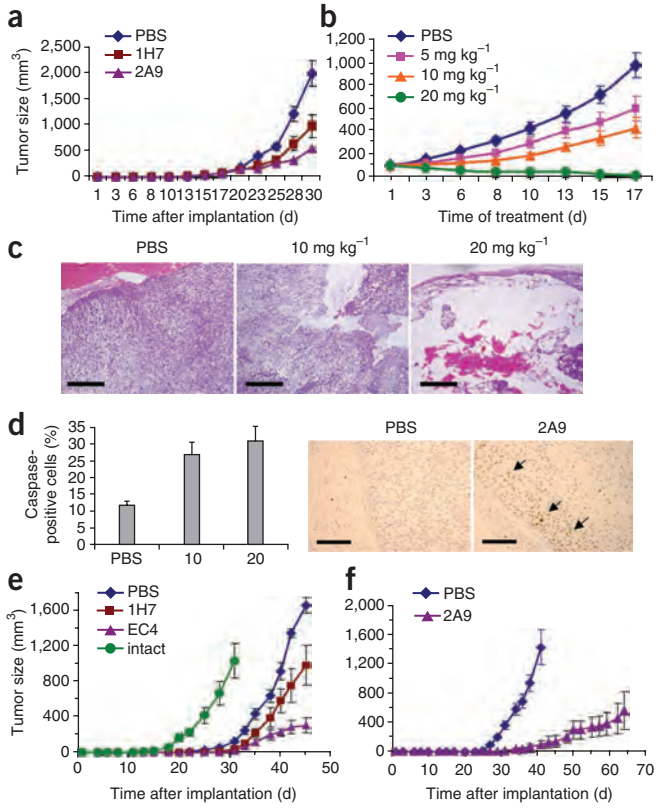

Figure 5 N-cadherin antibodies inhibit growth of established tumor and block progression to castration resistance *in vivo*. (**a**) Growth inhibition of established LAPC9-CR tumors (100 mm^3) upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight. $P = 0.003$ for both antibodies compared to control (PBS) group at 30 d. (**b**) Growth inhibition of established PC3 tumors (100 mm^3) 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 μm. (**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 ± 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-passage 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[14]. 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-β1 (TGF-β1), TGF-β2 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[15]. TGF-β can induce EMT and might mediate N-cadherin signal transduction. TGF-β, IL-6 and IL-8 have all previously been implicated in CRPC[16,17]. Silencing of N-cadherin in PC3 cells decreased IL-6, IL-8, vimentin, TGF-β 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[15]. 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

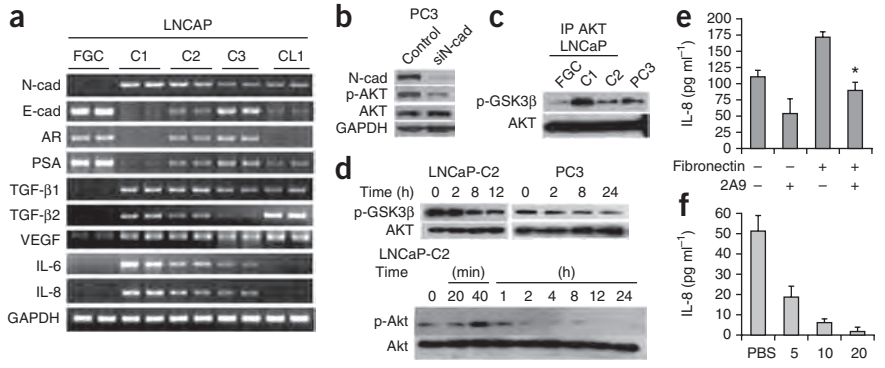

Figure 6 Gene expression change in N-cadherin–overexpressing cells. (**a**) RT-PCR analyses of gene expression in LNCaP cells without (FGC) or with high (C1, C2 and CL-AI) and low (C3) levels of N-cadherin. (**b**) Western blot of PC3 cells upon N-cadherin siRNA silencing (siN-cad). (**c**) AKT kinase 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 μg ml^{-1} 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 μg ml^{-1} 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 ± 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[18] and in 65% of tumors with Gleason score of ≥ 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[20]. 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[21]. 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**)[22]. 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-β, 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[16]. It has been shown that introduction of IL-8 leads to a decrease or loss in androgen receptor expression[16], 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[23]. 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[24]. 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[25]. 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/.

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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[14] 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[26] (Taconic).

Antibodies. Western blot analysis was performed as previously described[27] 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[28], 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^{low} 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[26] and screened by ELISA[13] 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^{-1} 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[26], in the presence of PBS control or 80 μg ml^{-1} 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 × 10^6 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[26]. We injected 1 × 10^6 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^3 in size or 200 mm^3 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 LAPC-9CR 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 × 10^6 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^{-1} 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. $P \leq 0.05$ was considered significant.

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doi:10.1038/nm.2236