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

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### 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

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## Abstract

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 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 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.

### 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 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<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 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. <sup>19</sup>). 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/>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

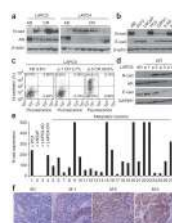
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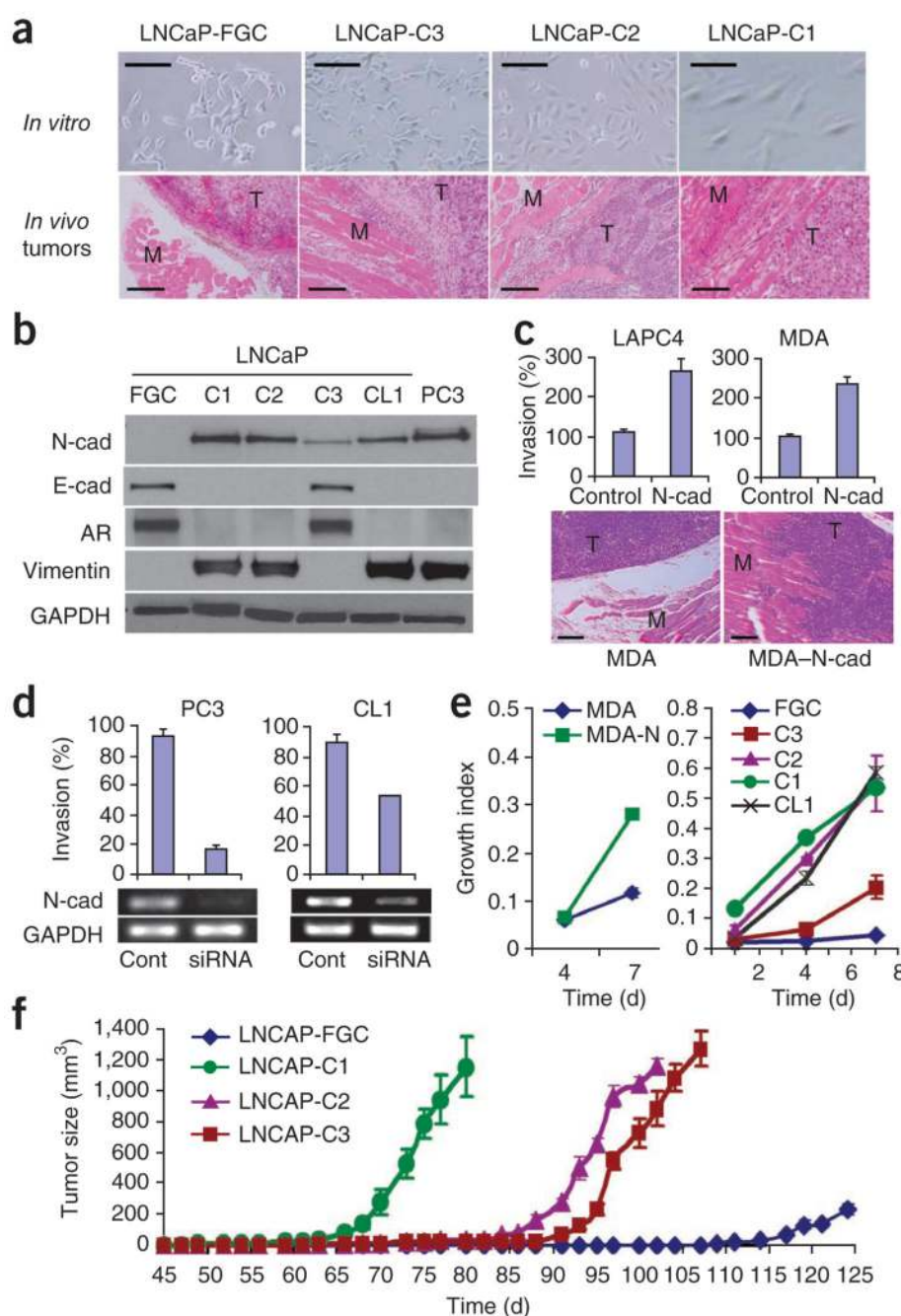


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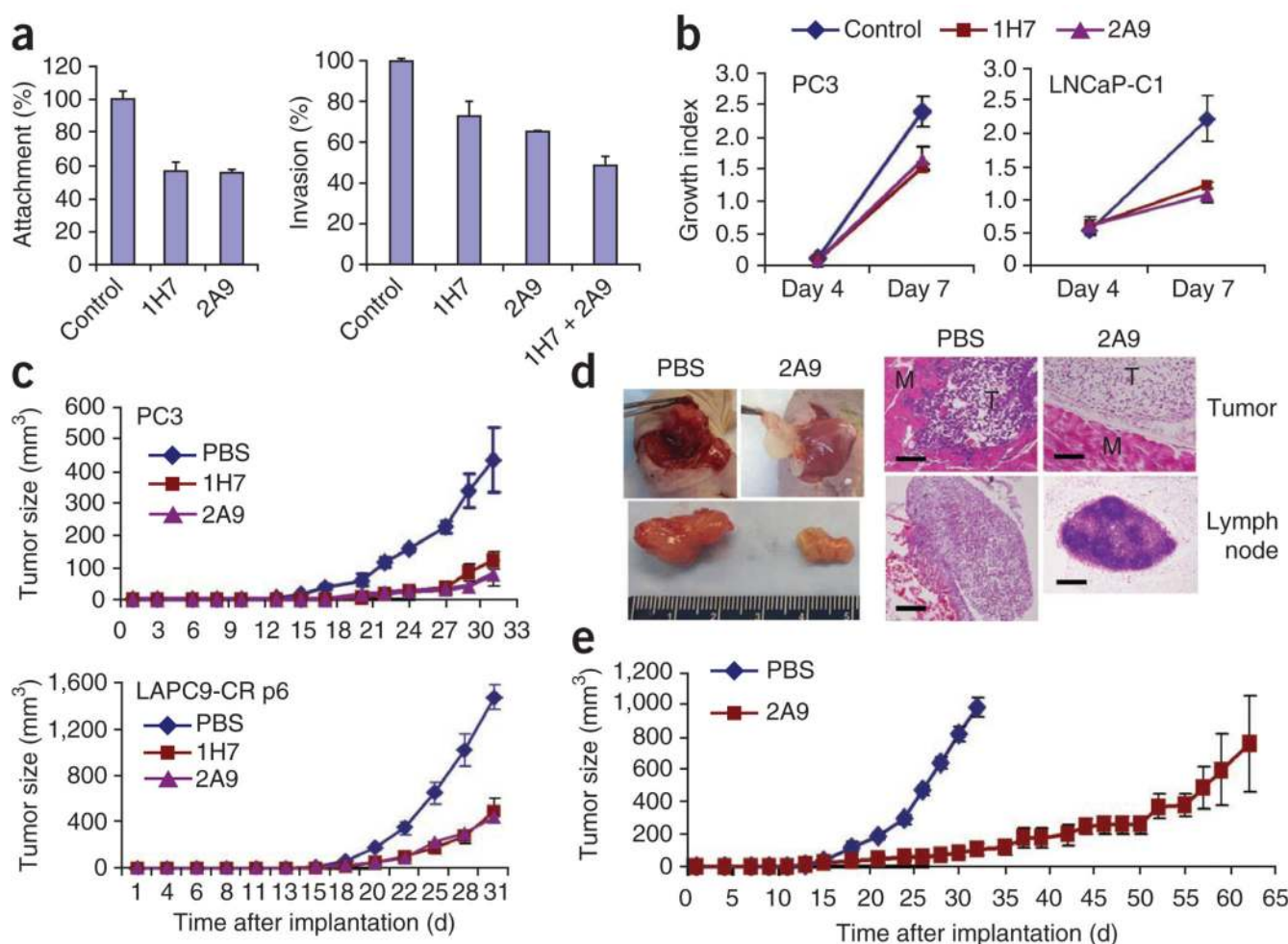
**Figure 1.**

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.



**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-

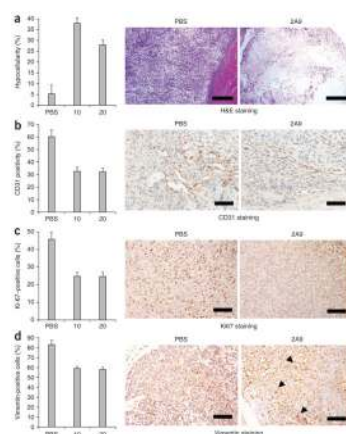
Pca-2b ( $P = 0.016$ ) cells ectopically overexpressing N-cadherin. Bottom, deep muscle invasion of *in vivo* MDA-N-cadherin tumor versus noninvasive MDA tumor (control). Scale bar, 100  $\mu$ m. M, muscle; T, tumor. **(d)** Invasion assays in endogenous PC3 and CL1 cells upon N-cadherin silencing by 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  $\pm$  s.e.m.



**Figure 3.**

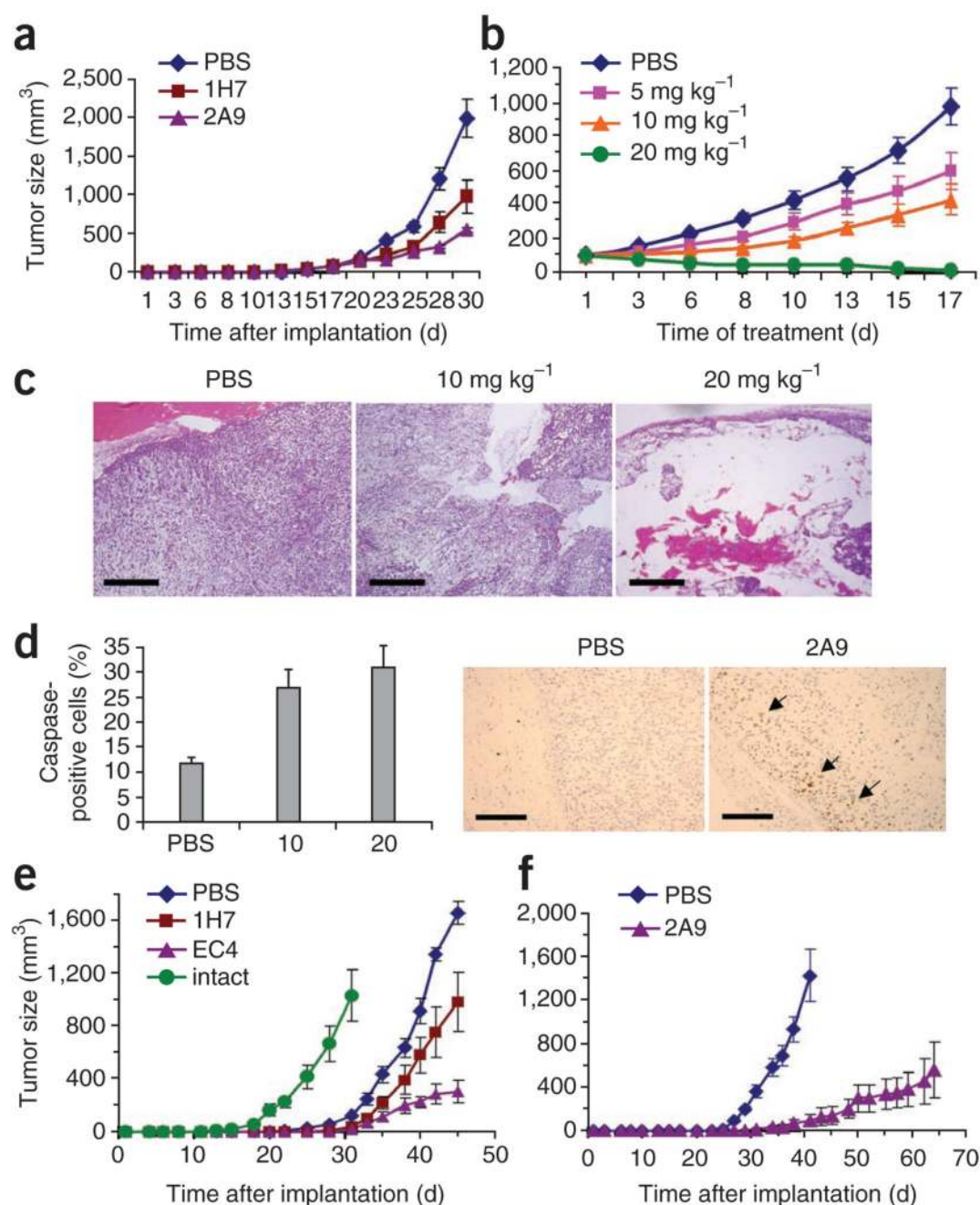
Antibodies against N-cadherin decrease invasion and tumor growth. **(a)** Attachment ( $P = 0.004$ ) and invasion ( $P = 0.05$ ) assays of PC3 cells upon treatment without or with 1H7 or 2A9 ( $80 \mu\text{g ml}^{-1}$ ). **(b)** Decrease in cell growth measured by proliferation assays of PC3 ( $P = 0.014$ ) and LNCaP-C1 ( $P = 0.01$ ) cells, upon treatment without or with  $80 \mu\text{g ml}^{-1}$  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  $10 \text{ mg per kg body weight}$ , 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,  $200 \mu\text{m}$ ) and metastases to axillary lymph nodes (scale bar,  $500 \mu\text{m}$ ). **(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.





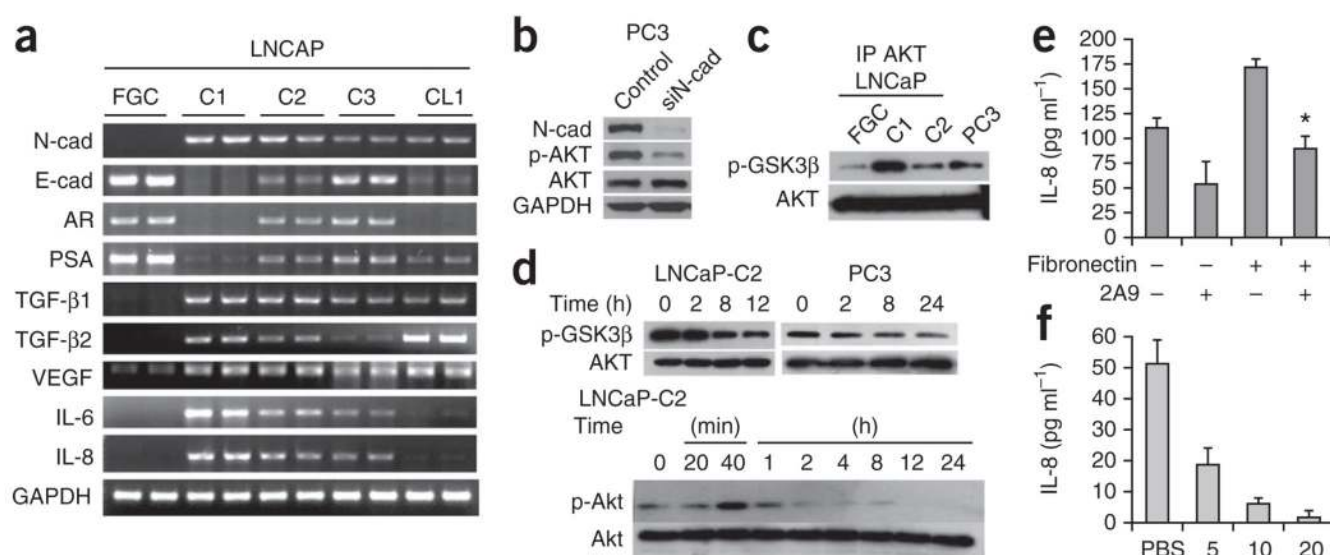
**Figure 4.**

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  $\mu\text{m}$ ) **(b)**; Ki-67 staining ( $P = 0.002$ ; scale bar, 500  $\mu\text{m}$ ) **(c)**; vimentin-positive regions (20 mg per kg body weight dose,  $P = 0.032$ ) **(d)**. The arrowhead points to stained cells. Scale bar, 500  $\mu\text{m}$ . 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 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  $\pm$  s.e.m.

**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  $\mu\text{g ml}^{-1}$  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  $\mu\text{g ml}^{-1}$  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  $\pm$  s.e.m.