Signal Transduction

Genetic Ablation of the Tetraspanin CD151 Reduces Spontaneous Metastatic Spread of Prostate Cancer in the TRAMP Model

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Abstract
Tetraspanins are integral membrane proteins that associate with motility-related molecules such as integrins. Experimental studies have indicated that they may be important regulators of tumor invasion and metastasis, and high expression of the tetraspanin CD151 has been linked to poor prognosis in a number of cancers. Here, we show for the first time that genetic ablation of CD151 inhibits spontaneous metastasis in a transgenic mouse model of de novo tumorigenesis. To evaluate the effects of CD151 on de novo prostate cancer initiation and metastasis, a Cd151–/– (KO) murine model was crossed with the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model. Mice were analyzed for initiation of prostate tumor by palpation and primary tumors were analyzed by immunohistochemistry. Liver and lungs were examined for incidence and size of spontaneous metastatic lesions by histopathology. Knocking-out Cd151 had no significant effect on prostate cancer initiation or on expression of markers of proliferation, apoptosis, or angiogenesis in primary tumors. However, it did significantly decrease metastasis in a site-specific fashion, notably to the lungs but not the liver. Thus, CD151 acts principally as promoter of metastasis in this model. Prostate cancer is the second highest cause of cancer-related deaths in men in most Western countries, with the majority of deaths attributed to late-stage metastatic disease. CD151 may prove to be a valuable prognostic marker for treatment stratification and is a possible antimetastatic target. Mol Cancer Res; 11(1); 95–105. ©2012 AACR.

Introduction
Prostate cancer is the most commonly diagnosed cancer in developed countries and the second most common cause of deaths in males. Because of the advent of prostate-specific antigen (PSA) screening, approximately 90% of patients still have the cancer confined to the prostate gland at diagnosis (1). The majority of deaths from prostate cancer are attributed to the incurable, late-stage, metastatic form of the disease (2). The molecular mechanisms that drive the metastatic cascade in prostate cancer are poorly understood although altered expression of various genes are known to influence metastasis (3). Furthermore, prostate cancer remains somewhat indolent in some patients while others develop aggressive metastatic forms of the disease. Understanding the molecules that influence the metastatic cascade may prove beneficial as prognostic markers and for patients diagnosed with early-stage cancer. This would allow stratification of treatment modalities that can have deleterious side effects.

The tetraspanin family of proteins comprises membrane-bound proteins that function by binding with and organizing other membrane-bound proteins such as integrins, growth factors, signaling molecules, and other tetraspanins to form multimolecular complexes termed tetraspanin-enriched microdomains (TEM). Through these interactions, tetraspanins have been shown to play a role in a variety of cellular processes that are essential for cancer progression such as signaling, adhesion, and motility (4–6).

The tetraspan CD151 (previously also known as PETA-3 and SFA-1; refs. 7, 8) has been shown in clinical studies to have a positive correlation with poorer patient outcomes in a variety of cancers including prostate (9), lung (10), colon (11), breast (12, 13), renal (14) and endometrial cancers (15). Molecular studies involving CD151 with cancer progression and metastasis to date have focused on in vitro and grafting models. These studies have shown that knockdown/-out or mutation of CD151 decreases cell motility (16–22) and/or decreases experimental metastasis (23–26).

In this study, we analyzed the influence of endogenous CD151 on initiation and progression of prostate cancer by using a Cd151 KO murine model crossed with the transgenic Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model of spontaneously forming prostate cancer.
The Cd151 KO mouse shows only slight abnormalities on the C57BL/6 background, specifically defective wound-healing capabilities, impaired platelet function, and T-cell hyperproliferation in vitro (27, 28), while the FVB/N background animals also develop progressive kidney disease (29, 30). The TRAMP model was developed as a tool to understand prostate cancer. The model is characterized by an intact immune system and expression of the SV40 T/t antigens (T/t-ag) under control of the androgen-sensitive, rat probasin promoter (31, 32). It has been extensively characterized and shown to display a spectrum of de novo developing prostate cancer from prostatic intraepithelial neoplasia (PIN) to spontaneously forming metastatic disease that closely recapitulates human prostate cancer progression (33–35).

Here, for the first time, we report the effects of ablation of Cd151 in a spontaneously developing model of prostate cancer. It was shown that the ablation of Cd151 did not affect primary tumor initiation; however, it did significantly reduce the incidence of metastases.

Materials and Methods

Animal breeding

Animal maintenance was in accordance with the Animal Care and Ethics Committee at the Australian BioResources SPF animal breeding facility (Moss Vale, New South Wales). The TRAMP mice were maintained as heterozygous for the SV40 T-ag gene by crossing female TRAMP+/− mice (Jackson Labs, Bar Harbour, ME) on the C57BL/6 background with male C57BL/6 mice. The original Cd151+/− KO mice were generated on a C57BL/6 background (28) and subsequently backcrossed onto the FVB/N background for no less than 10 generations. The FVB/N KO strain was maintained as Cd151+/−; animals due to progressive kidney disease in the Cd151 null animals on the FVB/N background (29). To generate TRAMP+/−,+/−/Cd151+/+, +/+, +/− F1 experimental animals, firstly, the TRAMP female mice on the C57BL/6 background were crossed with male Cd151+/− mice on the C57BL/6 background (as shown in Supplementary Fig. S1). Then, female TRAMP+/−/Cd151+/+ progeny mice were crossed with male Cd151+/+ mice on the FVB/N background to create F1 experimental animals on a genetically homogenous C57BL6/FVB/N background. All mice were genotyped by PCR from DNA extracted from ear tissue as previously described for TRAMP (31) and Cd151 (28).

Mice monitoring and tissue collection

All animal monitoring and procedures were done with the approval and in accordance with the guidelines of the Animal Care and Ethics Committee at The University of Newcastle. Cd151+/+,+/−,−/− littersmates were cohoused, monitored blind with respect to genotype, and euthanized after they had a palpable prostate tumor. Lower urogenital tracts, including prostate (and any associated tumor), seminal vesicles, and bladder (drained) were removed and weighed, fixed in 10% neutral buffered formalin (NBF; Clinipure) for 24 hours, then transferred to 70% ethanol until processed or placed in optimal cutting temperature (OCT) compound (Tissue-Tek) and snap frozen in liquid nitrogen. The lungs were inflated with 10% NBF via the trachea and removed together with the median lobe of the liver and placed in 10% NBF.

Histopathology and immunohistochemistry

Tissue was processed in the LYNX II automated tissue processor (EMS); briefly: 1 × 20 minutes in 70% ethanol, 3 × 20 minutes in 100% ethanol, 3 × 20 minutes in xylene, all with agitation, and 2 × 30 minutes with agitation and vacuum in melted paraffin wax (Meditex) and embedded in paraffin. The formalin-fixed paraffin-embedded (FFPE) tissues were sectioned at a thickness of 5 μm on a manual rotary microtome and adhered to slides coated with 3-amino propyl triethoxysilane (Sigma Aldrich). Sections were cleared in xylene, rehydrated in ethanol, and hematoxylin and eosin (H&E) staining was undertaken for histopathology. For immunohistochemistry, the indirect peroxidase method was used in conjunction with the Vectorstain ABC elite kits (Vector Labs) according to the manufacturer’s recommendations. Antigens were unmasked by heating the sections in citrate buffer (10 mm, pH6; Vector Labs) for 10 minutes at approximately 98°C. Primary antibodies used were mouse anti-SV-40 large T-antigen, clone P101 (1:400; BD Biosciences), mouse anti-bovine synaptophysin, clone SY-38 (prediluted; Chemicon), rabbit anti-human Ki67, clone SP6 (1:200; Neomarkers), rabbit anti-cleaved caspase-3, clone AS175 (1:800; Cell Signaling), rat anti-mouse CD31, clone SZ31 (1:20; Dianova), and mouse anti-human E-cadherin, clone 36 (1:1,000; BD Biosciences). For mouse antibodies, the “mouse on mouse” kit (M.O.M; Vector Labs) was used to eliminate intra-species reactivity. All washes and reagents, where applicable, were made up in PBT [1× phosphate-buffered saline, 1% BSA (w/v), and 0.1% Tween 20 (v/v)]. Labeling was detected with liquid chromogen diaminobenzidine, (DAB Impact; Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated with ethanol, cleared with xylene, and mounted with Cyto-seal 60 (Vector Labs). Isotype-matched antibodies were used as negative controls.

Immunofluorescence

Fresh-frozen blocks were sectioned at 5 μm on a cryostat, adhered to poly-lysine-coated slides and stored at −80°C until stained. For immunofluorescence labeling, sections were air dried and equilibrated to room temperature for 20 minutes, fixed in acetone for 10 minutes, and again air dried for 5 minutes, then rehydrated in PBS. Sections were blocked in 7% donkey serum (in PBT) for 30 minutes. The rabbit anti-mouse CD151 primary antibody, LAI-2 (29) was added to the section at 1:500 dilution in PBT for 1 hour and washed off with PBS. The anti-rabbit secondary antibody, conjugated to the Alexa 555 fluorophore (Invitrogen) was diluted at 1:400 in PBT and incubated for 1 hour, then washed in PBS. Sections were counterstained with DAPI and mounted in with Prolong Gold (Invitrogen). Cd151 KO
mouse tissue sections labeled with the LAI-2 antibody together with isotype-matched antibodies on normal mouse tissue were used as negative controls. Immunofluorescent slides were viewed with a Zeiss Axiosplan 2 upright fluorescence microscope (Carl Zeiss) using the various preset channels to excite the Alexa 555 fluorophore and DAPI. Capture of the images was done via a Zeiss Axiosplan MRm3 camera mounted on the microscope and operating through the Axiovision LE imaging system software (Version 4.8.1.0) at set exposure times of 500 milliseconds.

Quantitation of proliferation, apoptosis, and angiogenesis
Sections of poorly differentiated primary prostate tumors were stained with anti-Ki67, anti-active caspase-3, and anti CD31 as described earlier. Sections were scanned into digital format via the Aperio digital pathology system (Aperio). The digital images were viewed through Imagescope at ×200 magnification, and the Aperio positive pixel count and nuclear stain algorithms were used to quantify the immuno-histochemical (IHC) staining.

Quantitation of lung and liver metastases
The FFPE blocks containing the liver and lung tissue samples were sectioned at 5-µm thickness at 100-µm intervals; a total of at least 3 sections per organ were mounted onto silanized slides and stained by H&E. The glass slides were scanned into digital format via the Aperio digital pathology system (Aperio). The digital images were viewed through Imagescope at ×200 magnification, and the detection of at least 1 metastasis was considered positive for metastases. A representative slide for each organ from each animal was annotated in Imagescope around the lesions to gain number of lesions.

Statistical analysis
Statistical analysis of the Kaplan–Meier survival curves was done with the log-rank (Mantel–Cox) test. For binominal data such as incidence of metastases, contingency tables were created and the Fisher exact test was applied. Other data are shown as raw values and overlaid with the mean ± standard error of the mean or grouped arbitrarily (for illustration purposes; Fig. 7); however, statistical analysis to compare 2 groups (such as wt and KO) was done via the Mann–Whitney rank sum test on the raw values. Differences between groups was classed as significant when P was less than 0.05.

Results
CD151 ablation does not affect normal prostate development or transgene expression
The TRAMP-negative Cd151 wt and Cd151 KO F1 (C56BL6 xFVB/N) animals were analyzed for any developmental irregularities resulting from the Cd151 KO. All animals were fertile, offspring were generated at the expected genotypic ratios, and no overt irregularities in any organs were seen at autopsy. The prostates developed at similar rates between the Cd151 wt and KO animals, as can be seen Fig. 1A, at the whole (gross) and microscopic levels. None of the mice developed tumors by 40 weeks of age. Next, it was essential to investigate if the ablation of Cd151 in the TRAMP-positive animals affected the expression levels of the oncogenic SV40 T-ag transgene. As can be seen from Fig. 1B, expression of the SV40 T-ag protein was similar in both the TRAMP-positive Cd151 wt and Cd151 KO animals, in both normal prostate tissue and poorly differentiated primary prostate tumor tissue.

Expression patterns of CD151 in normal and cancerous mouse prostate tissue
Indirect immunofluorescence was used to analyze the expression patterns of CD151 in mouse prostatic tissue. The specificity of the rabbit anti-CD151 antibody was shown by the absence of labeling on Cd151 KO tissues (Fig. 2A and B) and in agreement with previous findings in our laboratory (29). Normal prostate glands were labeled for CD151, predominantly in the membrane and cytoplasm of the basal cells which surround the luminal secretory epithelial cells of the prostate gland. The glandular epithelial cells were labeled for CD151 in the basal and lateral membranes (Fig. 2C and D). In moderately/well-differentiated primary prostate tumors, expression of CD151 was membranous and cytoplasmic in the basal cells (where still present) and again in the basal and lateral membrane of the epithelial cells as represented in Fig. 2E and F. In poorly differentiated tumor tissue, CD151 was expressed in the membrane of the tumor cells in a heterogeneous focal manner.

Tumor progression in the model
The model showed a range of progression stages in the prostate tumors that included PIN (epithelial cells of glands displaying stratification, tufting with some cribriform structures, and elongated and hyperchromatic nuclei), well-differentiated (reduced glandular structure size and invasion of epithelial cells into the stroma, increased cribriform structures, and rounded nuclei compared with PIN), moderately differentiated (increased sheets of cells with infrequent irregular glandular structures) through to poorly differentiated primary prostate tumors (solid sheets of cancerous cells with very high nuclear to cytoplasmic ratios) as represented in Fig. 3A–H. Previous studies have reported that a subset of TRAMP animals on the FVB/N background have tumors with a neuroendocrine, rather than an epithelial, phenotype. The synaptophysin protein is the most widely used IHC marker of neuroendocrine cells. IHC labeling of synaptophysin on a section of mouse brain tissue (dentate gyrus) served as both a positive and negative control because of the neuroendocrine-rich and -negative layers (36, 37), and showed the predicted pattern of staining (Supplementary Fig. 2A and B). PIN and well-differentiated primary prostate tumor foci (Supplementary Fig. 2C), moderately differentiated metastatic lesions (Supplementary Fig. 2D), and metastatic lesions (Supplementary Fig. 2E) were all negative for the neuroendocrine cell marker, except for some unspecific labeling of prostatic glandular secretions. Staining...
for the epithelial cell marker E-cadherin revealed staining in normal prostate glands and PIN although this was lost in poorly differentiated tumor tissue (Supplementary Fig. S3).

**CD151 does not affect primary prostate tumor initiation**

After the animals had developed a palpable prostate tumor, they were culled and the lower urogenital tracts with associated tumors were removed and weighed. The time to palpable prostate tumor among the Cd151 wt, heterozygous, and KO animals was not significantly different (Fig. 4). Furthermore, as expected for this endpoint, there was no difference in the weights of the urogenital tracts/tumors among the experimental groups. At the endpoint, the number of animals in each group that developed de novo primary prostate tumors were as follows, 25/27 of the Cd151 wt animals, 19/21 of the Cd151 heterozygous animals, and 29/30 of the Cd151 KO animals. Of note is the n discrepancy in the tumor weights graph (Fig. 4A) of animals that developed primary prostate tumors, and the number of tumors weighed for each group was because the first 4 animals culled for palpable tumor did not have their tumors weighed as the protocols were developed. However, as appropriate, these animals were included in the survival curves as n reflects (Fig. 4B) and subsequent metastatic analysis.

**CD151 does not affect proliferation, apoptosis, or angiogenesis in the primary tumor**

The primary prostate tumors were further characterized by IHC for markers of proliferation (Ki67), apoptosis (active caspase-3), and angiogenesis (CD31). Figure 5 shows the specificity of the IHC staining for each marker together with the pseudo-markup of positively stained cells by the Aperio automated algorithms and graphical representation of the quantification. There were no significant differences among the Cd151 wt, heterozygous, or KO groups for any of the 3 markers.

**Metastatic lesions in the liver and lungs express the SV40 T-ag**

After the animals had developed a palpable primary prostate tumor, the liver and lungs were also collected and analyzed for metastases. Initially, the metastatic foci were characterized to establish that they did arise from the primary prostate tumor. The cancerous lesions in the liver and lungs were identified via H&E staining and were shown to label positively for the transgene protein SV40 T-ag (Fig. 6A). Two of the TRAMP-positive animals were culled due to sickness (14 and 18 weeks) and, on autopsy, were found to have extensive cancer lesions in the lungs and/or liver although they were shown to have only very early-stage prostate cancer, consisting of PIN. The liver and lungs lesions from these animals did not stain for the SV40 T-ag (Fig. 6A). This finding implies that the SV40 T-ag-positive tumors did not arise independently in the liver or lungs and are, indeed, derived from the primary prostate tumors. Furthermore, staining did not indicate unexpected expression of the transgene in normal tissue of the liver or lungs.

**Ablation of CD151 reduced the incidence of metastases to the lungs**

After the metastatic lesions in the liver and lungs had been shown to be derived from the primary prostate tumors, all livers and lungs were analyzed for presence or absence of metastases. From the mice that developed primary prostate tumors, the number of mice that progressed to have secondary metastatic lesions in either the liver and/or lung was...
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as follows for each group: Cd151 wt 13/24, Cd151 heterozygotes 4/13, and Cd151 KO 7/27. It should be noted the discrepancy of n between number of animals that developed primary tumors as stated above (Cd151 does not affect primary prostate tumor initiation) and total animals stated here was due to technical difficulties in fixing, processing, or sectioning of liver and/or lungs from a small number of animals for analysis of metastases. Exclusion was done with no knowledge of the donor animals’ genotype or presence or absence of metastases within those samples.

The datasets were grouped according to Cd151 wt, heterozygous, and KO genotypes and placed into contingency tables for statistical analysis via the Fisher exact test. Ablation of Cd151 was shown to significantly reduce the incidence of metastatic disease in the lungs, but not in the liver (Fig. 6C and D). Cd151 heterozygous animals appeared to have an intermediate incidence of lung metastasis, but the difference was not significant, with $P = 0.495$ for wt compared with heterozygous and $P = 0.225$ for heterozygous compared with KO. Furthermore, the ablation of Cd151 also significantly reduced the number and total area of metastatic foci to the lung, again with an intermediate effect seen in the Cd151 heterozygous animals and no significant difference in number or total area of metastatic foci in the liver (Fig. 7). The areas of the individual metastatic foci were also analyzed. Statistical analysis revealed no significant difference by a Mann–Whitney rank sum test between wt and Cd151 KO groups in the liver or the lung ($P = 0.208$ and $P = 0.37$, respectively; data not shown).

Discussion

Clinical studies have shown a positive correlation between Cd151 expression levels and progression in a number of cancers including prostate cancer. Experiments using in vitro and grafting techniques have shown that increased expression of CD151 is associated with increased motility and metastasis. In this study, we genetically deleted Cd151 in a transgenic model (TRAMP) that develops de novo prostate cancer and subsequent spontaneous metastatic lesions, to determine if the presence or absence of this tetraspanin influences initiation and/or progression of de novo prostate cancer in vivo.

In the prostate glands of wt animals, CD151 was expressed predominantly in the basal cells with staining also observed...
The ablation of Cd151 did not affect the expression of SV40 T-ag in normal prostate or tumor tissue in the model.

To address previously published reports of the TRAMP model on the pure FVB/N background developing a subset of aggressive cancerous lesions that display a neuroendocrine phenotype rather than epithelial as in human PCAs (39, 40), we used the neuroendocrine cell marker synaptophysin. Brain sections of the murine hippocampus, specifically the dentate gyrus, were used as both an experimental positive and negative control as this organ has layers that are mostly synaptophysin positive but also has distinct synaptophysin-negative layers (stratum pyramidal and granular cell layer; refs. 36, 37). Well-differentiated tumors were consistently negative for synaptophysin expression as were liver and lung metastases disseminated from primary prostate tumors. (Supplementary Fig. S2A). Our findings agree with the previously published report that the TRAMP model on the C57BL/6 × FVB/N F1 background does not display an overt neuroendocrine cellular phenotype in the early-stage prostate cancer development (34). The normal prostate glands highly expressed the epithelial cell marker, E-cadherin, with reduced but detectable expression in glands displaying PIN and little to no expression in poorly differentiated prostate tissue as expected (Supplementary Fig. S2B). These results, taken together, show that the TRAMP model on the F1 background does recapitulate the epithelial phenotype of human prostate cancer.

Almost all mice developed palpable tumors by 40 weeks of age (Fig. 4). The onset of primary prostate tumors (time to palpable tumor) was not affected by the ablation of Cd151 in this model. It is possible that the use of a more sensitive monitoring method might have revealed more subtle differences. We initially aimed to follow the tumor onset and progression by crossing in a luciferase reporter gene driven by a prostate-specific promoter and monitoring by noninvasive in vivo bioluminescence imaging as reported by Hsieh and colleagues (41). However, we were unable to obtain the necessary transgenic strain. Attempts to image tumor growth in the TRAMP model with fluorescent probes that are activated by the tumor-associated proteases, cathepsin, and matrix metalloproteinases were unsuccessful due to nonspecificity of the probes and poor localization to the tumor sites. Mice were euthanized as soon as palpable tumors were detected. Consistent with the lack of effect of Cd151 deletion on primary tumor growth, there were no significant differences between groups with regard to tumor weights or in their proportion of mitotic or apoptotic cells (Ki67- or activated caspase 3-positive, respectively; Fig. 5).

In vivo grafting experiments have been used by others to investigate the influence of CD151 on tumor growth at the primary site. Studies with syngeneic tumors grafted into Cd151 wt and KO mice have given variable results. Subcutaneous growth of Lewis Lung Carcinoma (LLC), but not B16 melanoma cells, was reduced in Cd151 KO mice (20, 26). This may be associated with an effect on tumor vascularization which was not seen in the case of B16 tumors.

**Figure 4.** Survival curves and tumor weights of Cd151 wt, heterozygous, and KO animals. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups.

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**Table 1:**

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<tr>
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**Figure 5:** Almost all mice developed palpable tumors by 40 weeks of age. The onset of primary prostate tumors (time to palpable tumor) was not affected by the ablation of Cd151 in this model. It is possible that the use of a more sensitive monitoring method might have revealed more subtle differences. We initially aimed to follow the tumor onset and progression by crossing in a luciferase reporter gene driven by a prostate-specific promoter and monitoring by noninvasive in vivo bioluminescence imaging as reported by Hsieh and colleagues (41). However, we were unable to obtain the necessary transgenic strain. Attempts to image tumor growth in the TRAMP model with fluorescent probes that are activated by the tumor-associated proteases, cathepsin, and matrix metalloproteinases were unsuccessful due to nonspecificity of the probes and poor localization to the tumor sites. Mice were euthanized as soon as palpable tumors were detected. Consistent with the lack of effect of Cd151 deletion on primary tumor growth, there were no significant differences between groups with regard to tumor weights or in their proportion of mitotic or apoptotic cells (Ki67- or activated caspase 3-positive, respectively; Fig. 5).

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**Graphical Abstract:**

A. Survival curves and tumor weights of Cd151 wt, heterozygous, and KO animals. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups. Littermate animals were cohoused and monitored, blind in regard to their genotype, for onset of a palpable prostate tumor. After animals from the endpoint study group had a palpable prostate tumor or were 40 weeks of age, they were culled and tumors of the urogenital organs including the prostates in the TRAMP-negative animals were removed together with the seminal vesicles and bladder (drained) and then weighed (A). The weights of the tumors were not statistically different among the wt, heterozygous, or KO groups (Mann–Whitney rank sum test). B, time in days until palpable tumor for the wt, heterozygous, and KO groups.
(26) or in primary tumors in our study (Fig. 5). This is discussed further in the following. Xenografts of tumor cells with or without CD151 knock-down (KD) by RNA interference into immunocompromised mice have shown reduced growth as a result of CD151 KD. Using MDA-MB-231 human breast cancer cells injected subcutaneously or into the mammary fat pad, Yang and colleagues (42) showed that KD of tumor cell CD151 substantially delayed tumor appearance although no difference in growth in vitro was observed. In addition, using MDA-MB-231 cells, Sadej and colleagues (12) showed reduced growth of CD151 KD subcutaneous xenografts in mice although, again, no intrinsic differences in growth in vitro were seen. Differences in the pattern of vascularization of CD151 KD tumors were noted (12). Taken together, the results indicate that CD151 on both the tumor cells and in the host environment can be involved in interactions promoting the growth of grafted tumors at the primary site. It will be important to examine further examples of de novo tumor development.

On dissection of animals with palpable prostate tumors, we frequently noticed macrometastases to the liver and, therefore, collected liver as well as lungs from all animals. The original TRAMP characterization articles did not commonly report liver metastases and seem to have set a trend to only examine the lungs for metastases in research involving this model (33, 34). We suggest that the liver should be included in future studies that use the TRAMP model to analyze metastasis. Although we saw frequent metastatic lesions in the liver, the ablation of Cd151 did not have a significant effect on the incidence of metastases to this organ. However, the ablation of Cd151 did have a significant effect on metastasis to the lungs of the animals, specifically, reductions in incidence, number, and total area of metastatic foci. The individual area of the foci in the lungs were not significantly different between the Cd151 wt, heterozygous, and KO groups, suggesting that ablation of Cd151 did not affect the growth of the foci after metastatic cells were recruited to the lungs. Overall, these data are consistent

Figure 5. Representative IHC labeling for markers of proliferation, apoptosis, and angiogenesis together with automated algorithm analysis of primary prostate tumors. The 5-μm FFPE tumor sections from animals culled with palpable prostate tumors were labeled by indirect peroxidase IHC with 3,3′-diaminobenzidine (DAB) as the chromagen using primary antibodies, anti-Ki67 (clone SP6; A), anti-active caspase-3 (clone ASP 175; D), and anti-CD31 (clone SZ31; G). Ki67 and active caspase-3 staining were analyzed for percentage of positive-staining cells using the Aperio nuclear algorithm (C and F, respectively), where the positive and negative cells were marked in yellow and blue (B and E), respectively. CD31 staining was quantified (H) using the positive pixel algorithm for percentage of positive pixels, marked in yellow, to negative pixels, which are marked in blue (H). The Mann–Whitney rank sum test was used to analyze the data among the Cd151 wt, heterozygous, and KO groups, in a pair-wise manner. No significant differences were seen, with all values larger than P, 0.279.

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with the view that CD151 is primarily a metastasis promoter (reviewed in ref. 6).

Multiple studies have shown that CD151 on tumor cells promotes their adhesion to basement membrane components, migration, and invasion in vitro (12, 42, 43), all of which are important in metastasis. Adhesion strengthening via laminin-binding integrins has been shown to depend on CD151 (44). The promigratory role of CD151 may depend, in part, on its regulation of integrin internalization and trafficking (16, 45). Sadej and colleagues (23) reported that KD of Cd151 depleted TGF-β-induced scattering of breast cancer cells in 3-dimensional culture.

The role of tumor cell CD151 in vivo has been investigated in models of spontaneous metastasis. Testa and colleagues (25) studied metastasis to the lung from cells of a human epithelial line placed onto the chorioallantoic membrane of chick embryos and were the first to show that CD151 is a potential metastasis-enhancer protein. They showed that a CD151 antibody, 1A5, did not affect proliferation of the tumor cells or primary tumor size but did significantly reduce metastasis. This group then went on to show that the CD151 antibody reduced metastasis by inhibition of cell detachment from the primary tumor and, therefore, entry into the vascular system (intravasation; ref. 24). Interestingly, they showed no difference in the recruitment of cancer cells to the lungs whether the CD151 antibody was used or not. This result appears to be at odds with other data which indicate involvement of CD151 at multiple steps of the metastatic cascade (described in the following), but may be explained by the different models used as well as the different modality of the CD151 interference. It is not clear precisely how MAb 1A5 is working, but it appears not to simply block CD151 function because KD of CD151 did not exhibit the same detachment defect. Rather, it is proposed that binding of MAb 1A5 mediates its effect by acting on CD151-containing complexes in the cell membrane (24).

In an earlier CD151 overexpression study, Kohno and colleagues (43) showed that CD151 promoted cell migration in vitro and metastasis to the lungs in mice following injection of cells via the orbital vein. Lung metastasis was markedly inhibited by pretreatment of the cells with a CD151 MAb. More recent studies have used tumor cells with KD of CD151. Sadej and colleagues (23) injected wt and CD151 KD MDA-MB-231 breast cancer cells into mice via the tail vein. The CD151 KD cells were shown to form lesser and smaller lung metastatic foci in comparison with the CD151 wt cells. The group then went on to investigate early recruitment of the Cd151 wt and KD cells to the lungs and liver over a 1-hour period via intravitral microscopy. They found a reduction in recruitment of the CD151 KD cells relative to wt cells to the lungs but, interestingly, not to the liver, which is in accord with our result on spontaneous metastasis. These findings indicate that CD151 on the primary tumor can influence metastasis by altering homing to secondary sites and also may account in part for the organ-site specificity we observed.
The studies reviewed above clearly show that tumor cell CD151 plays a major role in metastasis. The model used in our experiments had Cd151 knocked out in every cell of the animal, which included the environment (host) and the de novo-forming prostate tumor itself. Therefore, we cannot delineate whether the knockout of Cd151 in the host or tumor (or both) was responsible for the effects on metastasis we observed. CD151 is widely expressed in tissues, including the vascular endothelium, and reports using tumor grafts have indicated its importance in the host environment in regulating metastasis. Several of these have indicated effects on development of tumor vasculature. Although no vascular defects have been reported in unperturbed Cd151 KO mice (28, 30), a MAb to CD151 was previously shown to modify migration and in vitro tube formation of primary human endothelial cells (46).

Experiments using grafting of syngeneic tumors into wt and Cd151 KO C57BL/6 mice have shown effects mediated by CD151 in nontumoral tissues. Using LLC cells injected subcutaneously, Takeda and colleagues (20) showed reduced tumor growth and microvessel density in the Cd151 KO animals, indicating an effect on angiogenesis. This study included extensive characterization of endothelial cell function in wt and Cd151 KO mice in vitro and in vivo and showed that lack of CD151 resulted in impairment in several functions relevant to angiogenesis. The effect on tumor angiogenesis may be somewhat specific to different tumors because this group did not see an effect on subcutaneous growth or angiogenesis of another syngeneic tumor, B16 melanoma (26). Similarly, we did not see differences in blood vessel density in the primary prostate tumors in our experiments; however, more subtle differences in the tumor vasculature, possibly in tumor margins, may occur and might be detected by more detailed analysis. Importantly, the study by Takeda and colleagues (26) showed marked reduction in the number of lung metastases resulting from intravenous injection of both LLC and B16 melanoma cells in mice lacking CD151. Further experiments showed reduced tumor–endothelial adhesion and transendothelial migration as the likely mechanism. The results of these 2 studies clearly show the important role of endothelial cell CD151 in tumor growth and metastasis.

Studies with Cd151 KO mice have indicated that the protein plays important roles in platelet function and antigen presentation to T cells by dendritic cells (28, 47, 48), both of which have the potential to affect metastasis (reviewed in ref. 49). Takeda and colleagues (26) did not see any difference in aggregation of B16 melanoma cells between Cd151 wt and KO mice; however, the model may not accurately reflect the process of spontaneous metastasis, nor did preliminary investigations reveal any differences in antitumor immunity. In both cases, further studies are required.

From published studies reviewed above, it appears that CD151 on both the tumor cells and the host are involved in regulating tumor growth and metastasis. CD151 on the tumor cells may regulate cell–cell attachment and release from the primary tumor mass (24, 50) and influence extravasation through its well-established influence on adhesion and migration. In some systems, CD151 on tumor cells appears to regulate angiogenesis, which is likely to promote primary tumor growth as well as metastasis (12). Studies with Cd151-ablated mice indicated that, at least in some instances, angiogenesis is also regulated by CD151 expression on the vascular endothelium of the host animal (20). Many additional steps in the metastatic cascade, including cell binding to the vascular endothelium in the target organ and transmigration into the tissue, involve interactions with the vascular endothelium. Although vasculogenesis appears...
normal in Cd151-null mice, multiple assays showed endothelial functional defects which may influence pathologic angiogenesis as well as tumor cell homing to secondary sites (20). Indeed, the lack of CD151 in host mice led to reduced tumor-cell attachment to endothelial cells, transmigration, and metastasis to the lungs (26). Thus, the CD151 status of both tumor cells and environment is important for tumor growth and metastasis.

In our experiments, we found that deletion of CD151 on both the tumor and the host led to reduced spontaneous metastasis to the lung but not the liver. In an attempt to clarify the role of tumor versus environmental CD151, we conducted a preliminary experiment in which cells of a luciferase-transduced TRAMP cell line (Dr C. Power and Dr T-Z. Hung, University of New South Wales, unpublished data) were injected via the heart into (C57BL/6 x FVB/N) F1 wt and Cd151 KO mice. Animals were monitored by in vivo bioluminescence imaging over 4 weeks. The results were inconclusive because metastasis was predominantly to the liver, with some to bone, but few lung metastases were observed. No difference in the frequency of metastases to the liver between groups was observed. Further experiments using cultured primary tumor cells and different routes of injection need to be carried out.

In summary, here for the first time we report the effects of ablation of Cd151 in a spontaneously developing model of prostate cancer. It was shown that the ablation of Cd151 did not affect de novo primary tumor growth or angiogenesis; however, it did significantly reduce the incidence, number and total area of spontaneously forming metastatic foci. Understanding molecules that are involved in the metastatic cascade in prostate cancer is of great importance, for 2 main reasons. Firstly, current treatment options for hormone-refractory metastatic prostate cancer are limited to palliative care and, secondly, the advent of PSA testing has seen a dramatic increase in the diagnosis of cancer localized to the prostate. Molecules such as CD151 may prove beneficial as prognostic markers to stratify which patients’ cancer would remain indolent and which would go on to more advanced disease, to allow the application of appropriate personalized treatment modalities.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: L.K. Ashman

Development of methodology: B.T. Copeland

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.T. Copeland

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.T. Copeland

Writing, review, and/or revision of the manuscript: B.T. Copeland, L.K. Ashman

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.T. Copeland, M.J. Bowman

Study supervision: L.K. Ashman

**Acknowledgments**

The authors thank Matthew Nayler for his advice on the TRAMP model, Ricardo Vázquez for his assistance with pathology issues, and Patrick McElhuff for statistical advice. Thanks also go to Nikkii Verrills and Severine Roselli for their insights and helpful comments on the manuscript. The authors also thank Carl Power and Tzung-Tyng Hung for providing the cardiac injection metastatic model and kind assistance with the bioluminescence imaging.

**Grant Support**

This work was financially supported by The Cancer Council New South Wales (grant 0188877). B.T. Copeland was supported by a University of Newcastle PhD scholarship and The Hunter Medical Research Institute, Mary Minto Sawyer Scholarship (grant 0188877). B.T. Copeland was supported by a Principal Research Fellowship from the National Health and Medical Research Council of Australia.

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Received August 2, 2012; revised October 14, 2012; accepted October 22, 2012; published OnlineFirst November 6, 2012.

**References**


Reduced Metastasis of de novo Prostate Cancer in Cd151 KO Mice


Genetic Ablation of the Tetraspanin CD151 Reduces Spontaneous Metastatic Spread of Prostate Cancer in the TRAMP Model

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