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TITLE: The Role of Retinal Determination Gene Network (RDGN) in Hormone Signaling Transduction and Prostate Tumorigenesis

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Prostate cancer is the most frequent malignancy and the second leading cause of cancer-related death among men in the United States. Based on the unique characteristics that those prostate epithelia are dependent on androgen for growth and survival, androgen deprivation therapy (ADT) has been a first choice of prostate cancer treatment. Despite early success to suppress prostate tumor growth, ADT eventually fails leading to recurrent tumor growth in a hormone-refractory manner, even though AR remains to function in hormone-refractory prostate cancer. We identified a member of androgen receptor (AR) co-regulator, named dachshund (dac). dac was originally discovered as a dominant inhibitor of hyperactive growth factor receptors in genetic screens. RDGN pathway, consisting of the dachshund (dac), eyes absent (eya), eyeless, and sine oculis (so) (Six) genes DACH1 is structurally distinct to the current known tumor suppressors. We propose this unique function as a new type of tumor suppressor and refer to DACH1 as a “commandeering tumor suppressor.” Recently, we reported that expression of DACH1 is lost in human prostate cancer tissues and restoration of DACH1 inhibited ligand induced AR activity. Although the abnormal expressions of RDGN genes have been reported in prostate cancer, the precise role of RDGN in prostate cancer is not clear.
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INTRODUCTION

Prostate cancer is the most frequent malignancy and the second leading cause of cancer-related death among men in the United States. Based on the unique characteristics that those prostate epithelia are dependent on androgen for growth and survival, androgen deprivation therapy (ADT) has been a first choice of prostate cancer treatment. Despite early success to suppress prostate tumor growth, ADT eventually fails leading to recurrent tumor growth in a hormone-refractory manner, even though AR remains to function in hormone-refractory prostate cancer. We identified a member of androgen receptor (AR) co-regulator, named dachshund (dac). dac was originally discovered as a dominant inhibitor of hyperactive growth factor receptors in genetic screens. RDGN pathway, consisting of the dachshund (dac), eyes absent (eya), eyeless, and sine oculis (so) (Six) genes DACH1 is structurally distinct to the current known tumor suppressors. We propose this unique function as a new type of tumor suppressor and refer to DACH1 as a “commandeering tumor suppressor.” Recently, we reported that expression of DACH1 is lost in human prostate cancer tissues and restoration of DACH1 inhibited ligand induced AR activity. Although the abnormal expressions of RDGN genes have been reported in prostate cancer, the precise role of RDGN in prostate cancer is not clear.
Aim 1. Evaluate the physiological role of DACH1 in prostate gland development and ErbB2-induced prostate tumor. Investigating the role of DACH1 as a physiological co-repressor of AR will be conducted on transgenic mice in which the Dach1 gene is flanked by loxP sites (Dach1fl/fl) and crossed with Probasin-Cre (Pb-Cre) to generate double transgenic mice, those mice will be crossed with Probasin-erbB2Δ (Pb-erbB2) transgenic mice to create triple transgenic mice, Dach1fl/fl/Pb-Cre/ Pb-erbB2Δ.

Using Dach1fl/fl/Probasin-Cre bi-transgenic mice endogenous Dach1 was shown to serve as a key endogenous restraint to prostate epithelial cell growth, and migration.

Conditional Dach1 gene knockout in the prostate demonstrates a role for endogenous Dach1 as an inhibitor of cellular proliferation and inducer of apoptosis. In order to determine the potential role for Dach1 in prostatic cellular proliferation in vivo, bi-transgenic mice were generated. The Dach1fl/fl mice were intercrossed with Probasin-Cre mice, in which Cre recombinase is expressed in the basal and luminal epithelial cells of the prostate. Immunohistochemical staining demonstrated the loss of Dach1 in both the anterior and ventral prostates of the bi-transgenic mice. An analysis of cellular proliferation and apoptosis was conducted. Ki67 staining, a surrogate measure for cellular proliferation, was significantly increased in the PEC of the Dach1fl/fl/Probasin-Cre mice (Fig. 1). TUNEL staining was conducted to assess the effect of endogenous Dach1 on cellular survival in vivo. The percentage of apoptotic cells was reduced ~3-fold in the Dach1−/− prostate (Fig. 2). Consistent with the finding that DACH1 inhibited cyclin A2 and cyclin E1, immunohistochemical staining demonstrated that the abundance of cyclin A2 and cyclin E1 was increased in the Dach1−/− PEC.
In order to determine whether DACH1-regulated prostate cancer cellular migration, the effect of DACH1 on the ability of PC3 cells to traverse a membrane was assessed. DACH1 expression in PC3 cells reduced transwell migration by >90% (Fig. 3A, B) and deletion of the DACH1 DS domain abrogated the effect on cell migration (Fig. 3B). In order to define further the distinct components of cell migration regulated by DACH1 expression, video microscopy was conducted to determine the distance and effect of DACH1 on prostate cancer cellular migratory directionality. DACH1 inhibited the distance traveled by PC3 cells. The effect was abrogated by the deletion of the DS domain (Fig. 3C). The velocity of migration was also reduced by DACH1 requiring the DACH1 DS domain (Fig. 3D).

In order to define the mechanisms by which DACH1 inhibited cellular migration, microarray analysis was conducted of key growth factor receptor and cytokine/chemokine signaling pathways known to promote cellular proliferation and migration. A module of secreted cytokine signaling was selectively repressed by DACH1, including IL-6, IL-8, and CXCL6, 1, 2, 5 and IL-6 (Fig. 3E). Deletion of the DS domain abrogated DACH1-mediated repression of IL-8 and IL-6 expression (Fig. 3E).

In order to determine the mechanisms by which DACH1 reduced the cytokine signaling module, we considered the possibility that DACH1 may inhibit the transcription of CXCL genes. We examined the IL-6 and IL-8 promoters. Both IL-6 and IL-8 were repressed in a dose-dependent manner by DACH1, and repression required the DS domain (Fig. 3F, G).

In order to determine whether DACH1-mediated repression of IL-8 was oncogene specific, we assessed the isogenic oncogene transformed prostate cancer cell lines, in which DACH1 expression was reduced. Microarray analysis of the isogenic prostate cancer cell lines in tissue culture (Fig. 4A) or in vivo in tumors derived from FVB mice, demonstrated increased expression of IL-6 and IL-8 (CXCL1) in each of the lines transformed by distinct oncogenes (c-Myc, NeuT, H-Ras, V-Src). Immunohistochemical staining of the extirpated tumors demonstrated a significant inverse correlation between DACH1 and the abundance of CXCL1 (KC), IL-6 and CXCR2 (the receptor for CXCL1) (Fig. 4C-E).

**Figure 3. DACH1 inhibits transwell migration and cytokine gene expression via the DS domain.** A) DACH1 were Stained after transwell migration. PC3 cells stably expressing either vector, DACH1 or ΔDS. B) The relative migration is shown for N>5 separate experiments. C) The PC3 stable cell lines were analyzed by video microscopy for distance traveled. Representative examples of individual cell tracking are shown for stable cells co-incubated with the media from either vector expressing DACH1, or expressing DACH1 ΔDS in PC3 cells. D) The cellular velocity for the PC3 cells is shown as mean ±SEM for three separate experiments. E) Microarray analysis of PC3 cells stably expressing either DACH1, DACH1 ΔDS or vector control were analyzed for the relative abundance of growth factor or chemotactic gene mRNA. DACH1 inhibits CXCL gene expression as shown by the blue color indicating repression in the presence of DACH1 expression. F) The relative luciferase reporter activity of the IL-6 and G) IL-8 promoter was normalized as one for the vector control. The luciferase promoter reports were transfected into PC3 cells, together with equimolar amounts of either vector, DACH1 expression vector or the ΔDS expression vector. Data are ±SEM for N>5 separate experiments.
**Dach1** inhibition of cellular migration involves secreted cytokines (IL-6, CXCL1). In order to determine whether endogenous DACH1 regulated the secretion of the cytokine signaling mRNA module identified in human prostate cancer cells in tissue culture, the prostatic epithelium of the bi-transgenic mice (Dach1fl/fl/probasin Cre) was analyzed.

**Aim 2. Examine the role of DACH1 in prostate cancer cell AR signaling transduction, proliferation, migration and invasion in vitro:**

DACH1 inhibited androgen therapy-resistant prostate cancer (CRPC) tumor growth in mice and contact-independent growth, via a helix-turn-helix DNA interaction domain which was required to both repress CRPC growth and inhibit expression and secretion of a CXCL gene cluster (IL-8, IL-6).

**Figure 4. Dach1 expression is reduced in oncogenic transformed PEC, and inverse PEC co-related with cytokine production.** A) Microarray based gene expression of cytokines / chemokines in isogenic PEC in tissue culture or B) from tumors grown in FVB mice. The relative expression levels are shown in C-E. F) Relative DACH1 mRNA levels were determined from Oncomine in either prostate cancer samples or benign prostate disease. DACH1 levels are significantly reduced in metastatic prostate cancer samples.

**DACH1 inhibits CRPC contact-independent growth.** In order to define the genetic cell-cycle targets of DACH1, and to determine whether DACH1 was capable of inhibiting AR negative prostatic cancer cell contact-independent growth, the PC3 cell line was stably transduced with an expression vector encoding DACH1, or a mutant of DACH1 deleted of the DS domain. Microarray analysis was conducted of the cell lines (Fig. 5A). Interrogation of the cell cycle control proteins demonstrated that, unlike several other cell types in which cyclin D1 is a direct-target of DACH1 repression, the cyclin E1 and cyclin A2 gene were repressed in PC3 cells (Fig. 5A). Deletion of the DS domain abrogated repression of cyclin A2 mRNA expression (Fig. 5A). We examined the possibility that the cyclin E1 and cyclin A2 genes were direct transcriptional targets of DACH1. The cyclin E1 and cyclin A2 promoter linked to a luciferase reporter gene was examined for responsiveness to DACH1. Transfection of a DACH1 expression vector with either the cyclin E1 or cyclin A2 promoter-reporter in PC3 cells induced a dose-dependent repression dependent upon the DS domain (Fig. 5B, C). Antibodies directed to the DACH1 protein demonstrated the presence of DACH1 in PC3 cells stably expressing DACH1 or the ΔDS domain (Fig. 5D).
Aim 3: Investigate the role of DACH1 in tumor growth in vivo using xenograft models.

DACH1 inhibition of prostate cancer cellular invasion and migration required CXCL gene expression. DACH1 expression was reduced in metastatic human prostate cancer.

DACH1 inhibits CRPC and PEC growth in vivo. In order to determine whether DACH1 inhibited CRPC tumor growth in vivo, tumor size of DACH1 transduced PC3 cells was determined weekly after implantation (Fig. 2A). The tumor size increased in the vector control, but was reduced by the expression of DACH1, with an approximately 50% decrease in tumor volume at week 7. Deletion of the DS domain abrogated the reduction of tumor growth in nude mice. The tumor weight was similarly reduced, approximately ~50% in vivo (Fig. 6B), whereas deletion of the DS domain abrogated the inhibition of tumor weight. The CWR22Rv1 line was derived from a relapsed human prostate cancer CWR22 xenograft (1). This line encodes a ligand independent AR variant that arises by splicing of cryptic exons (2). The CWR22Rv1 cells express the AR variant and are p53 positive, unlike PC3 cells, which are AR negative and p53 negative. Re-expression of DACH1 in CWR22Rv1 cells reduced tumor growth in mice approximately 50% at 5 weeks post implantation (Fig. 2C). Deletion of the DACH1 DS domain reduced, but did not abrogate the inhibition of cell growth, suggesting distinguishable domains of DACH1 may be involved in the inhibition of cellular growth in AR negative vs. AR positive cells in vivo. The weight of the tumor was reduced >80% by DACH1 expression (Fig. 2D, E).
Aim 4. Analyze the expression of DACH1, Eya1 and Six1 in human prostate tumor samples.

In order to determine whether a clinical correlate existed for DACH1-mediated repression of cellular migration, we hypothesized that DACH1 expression may be lost in metastatic prostate cancer. Interrogation of clinical databases demonstrated the relative abundance of DACH1 was reduced in prostate cancer compared with benign prostate disease, with significant further reduction in metastatic prostate cancer samples (Fig. 4F) (above).

Figure 6. DACH1 inhibits prostate cancer tumor growth and cellular proliferation in mice. A-B) PC3 cells stably transduced with either control vector DACH1 or the DACH1 ΔDS mutant were introduced into nude mice and assessed by A) tumor size or B) tumor weight after implantation C-D) 22RV1 cells stably expressing either DACH1, DACH1 ΔDS or vector control were assessed after implantation into mice. C) The tumor size or D) the tumor weight representative examples of the tumors after extirpation was determined. The data are shown as mean ±SEM for n>7 throughout.
KEY RESEARCH ACCOMPLISHMENTS

1. Conditional *Dach1* gene knockout in the prostate demonstrates a role for endogenous Dach1 as an inhibitor of cellular proliferation and inducer of apoptosis.

2. DACH1 inhibits prostate cancer cellular migration and persistence of migratory directionality.

3. DACH1 inhibits CRPC and PEC growth *in vivo*.

4. *Dach1* inhibition of cellular migration involves secreted cytokines (IL-6, CXCL1).

5. DACH1 was reduced in prostate cancer compared with benign prostate disease, with significant further reduction in metastatic prostate cancer samples.
REPORTABLE OUTCOMES

Manuscripts


Abstracts and Presentations


Licenses applied for and/or issued

- Nil

Degrees obtained that are supported by this award

- Nil

Development of cell lines, tissue or serum repositories

- Nil

Informatics, such as databases and animal models, etc.

- Animal model first prostate specific Dach1 gene deletion mouse – Dach1^{fl/fl} Probasin Cre

Funding applied for based on work supported by this award

- Nil

Employment or research opportunities applied for and/or received based on experience/training supported by this award.

- Post-Doctoral training by Dr. Chen
CONCLUSION

Using \textit{Dach1}\textsuperscript{fl/fl}/Probasin-Cre bi-transgenic mice endogenous \textit{Dach1} was shown to serve as a key endogenous restraint to prostate epithelial cell growth, and migration.

These studies suggest that endogenous Dach1 is the major endogenous inhibitor of cytokine signaling. Cytokines and chemokines have broad effects on cellular inflammation, migration, invasion and growth. Inflammation is an important early change in human prostate cancer. Establishing that Dach1 is the key inhibitor of cytokine signaling in the prostate will therefore be an important next goal.

Furthermore, we will need to establish whether reduction of endogenous DACH1 occurs early in PIN lesions as humans as inflammation is a common early event in human PIN lesions.
REFERENCES
