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EXTRA VIEW

MYC and *PVT1* synergize to regulate RSPO1 levels in breast cancer

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ABSTRACT

Copy number gain of the 8q24 region including the v-myc avian myelocytomatosis viral oncogene homolog (*MYC)* oncogene has been observed in many different cancers and is associated with poor outcomes. While the role of *MYC* in tumor formation has been clearly delineated, we have recently shown that co-operation between adjacent long non-coding RNA plasmacytoma variant transcription 1 (*PVT1)* and *MYC* is necessary for tumor promotion. Chromosome engineered mice containing an increased copy of *Myc-Pvt1* (Gain *Myc-Pvt1*) accelerates mammary tumors in *MMTV-Neu* mice, while single copy increase of each is not sufficient. In addition, mammary epithelium from the Gain *Myc-Pvt1* mouse show precancerous phenotypes, notably increased DNA replication, elevated ϒ*-H2AX* phosphorylation and increased ductal branching. In an attempt to capture the molecular signatures in pre-cancerous cells we utilized RNA sequencing to identify potential targets of supernumerary *Myc-Pvt1* cooperation in mammary epithelial cells. In this extra view we show that an extra copy of both *Myc* and *Pvt1* leads to increased levels of *Rspo1*, a crucial regulator of canonical β-catenin signaling required for female development. Human breast cancer tumors with high levels of *MYC* transcript have significantly more *PVT1* transcript and *RSPO1* transcript than tumors with low levels of MYC showing that the murine results are relevant to a subset of human tumors. Thus, this work identifies a key mechanism in precancerous and cancerous tissue by which a main player in female differentiation is transcriptionally activated by supernumerary *MYC* and *PVT1*, leading to increased premalignant features, and ultimately to tumor formation.

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Introduction

The v-myc avian myelocytomatosis viral oncogene homolog (*MYC)* gene is one of the most studied oncogenes in the history of cancer research.[1] Though mutations in MYC are found with measurable frequency in human cancers,[2] MYC is commonly amplified in cancer as a part of the 8q24 amplicon.[3] We have recently shown that gain of the genomic region containing *Myc*, long non-coding RNA plasmacytoma variant transcription 1 *(Pvt1), Ccdc26 and GasderminC (GsdmC)* enhances tumor formation in mice compared to gain of an extra copy of Myc alone. Subsequently, we showed that *Pvt1/PVT1*co-operates with and potentiates *Myc/MYC*, in cancers with supernumerary MYC.[4] Chromosome engineering was used to generate sibling animals with a single extra copy of a genomic region and these animals allow the study of specific copy number gains in a clear and coordinated fashion along with control sibling mice.[5] Using this model we showed that a single copy of the 8q24.21 syntenic region containing both *Myc* and *Pvt1* is sufficient to promote tumorigenesis in the MMTV-*Neu* mouse, while an extra copy of *Myc* or *Pvt1* independently is not capable of this tumor promotion/acceleration.[4] Additionally, single copy number gain of the *Myc, Pvt1, Ccdc26* and *Gsdmc* region (hereafter anointed as *Gain(Myc-Pvt1)*) gives rise to a precancerous phenotype in mouse mammary tissues, notably increased DNA replication, elevated *γ-H2AX* foci, and significantly increased ductal branching which also was not observed in mice harboring an increase copy of *Myc* only (Gain (*Myc*)) or *Pvt1*, *Ccdc26*, *Gsdmc6* (hereafter anointed *Gain (Pvt1)*) alone (2). We hypothesized that a systematic analysis of the genes differentially expressed in the mammary epithelial cells from the Gain (*Myc+Pvt1*) relative to the Gain (*Myc*), Gain (*Pvt1*) and wild type (wt) siblings will reveal the molecular signatures associated with the precancerous lesions associated with the Gain (*Myc+Pvt1*) mammary glands. Here we report the analyses of RNA-SEQ data from primary mammary epithelial cells from these mice and compare the findings with transcriptome analysis of human breast cancer with increased MYC expression.

Transcriptome analysis reveals molecular signatures in precancerous mammary lesions with supernumerary Myc +Pvt1

We rationalized that the identification of genes from engineered mammary epithelial cells which are differentially expressed between the pre-tumorigenic state (*Gain(Myc-Pvt1)*) and each of the non-tumorigenic states (*Gain (Myc), Gain*

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(Pvt1), wild type (*wt*)) would be useful in understanding the early molecular mechanisms of breast cancer formation. To validate that the RNA-SEQ libraries were of sufficient quality for meaningful analyses we compared the FPKM levels obtained for Myc and Pvt1 across the 4 sample groups relative to the FPKM value obtained for ActB. Plots of the relative transcript levels show that Myc and Pvt1 levels are increased at expected levels in *Gain(Myc-Pvt1)* animals relative to the *other genotypes* (Fig. 1B). Because we could validate differential transcript levels within the region consistent with expectation, we concluded that we would be able to see differential expression in our data set. To look for differentially expressed genes we first carried out FPKM based analyses of the *Gain(Myc-Pvt1)* compared to the *wt, Gain (Myc)* and *Gain (Pvt1)* samples using CUFFLINKS. Dmbt1 and Lrcc15 transcript levels were observed by CUFFLINKS to be differentially expressed between *Gain(Myc-Pvt1)* and all other genotypes (Fig. 1C). DMBT1 transcript level is decreased in breast cancer[6] and DMBT1 has been described as a breast cancer susceptibility locus in mouse tumors.[7] Mutations in DMBT1 have been associated with increased breast cancer risk in human population.[8] Dmbt1 is almost completely lost in the *Gain(Myc-Pvt1)* but remains expressed in *wt, Gain (Myc)* and *Gain (Pvt1)* mammary epithelial cells. We also find strong enrichment of Lrcc15 transcript in *Gain(Myc-Pvt1)* but not in *wt, Gain (Myc)* and *Gain (Pvt1)* (Fig. 1C). Lrcc15 (LIB) is strongly expressed in breast cancer tumors.[9]Additional genes were identified with greater than 5-fold change in each of the comparisons above and were called as significantly different in 2 of the 3 comparisons using Cuffdiff. Rspo1 was observed to be an average of 8-fold induced in GainMyc-Pvt1 precancerous mammary epithelial cells relative to the tissues not exhibiting the premalignant phenotype (Fig. 1C). *Rspo1* belongs to the R-spondin family of proteins which are secreted agonists of the canonical Wnt/β-catenin

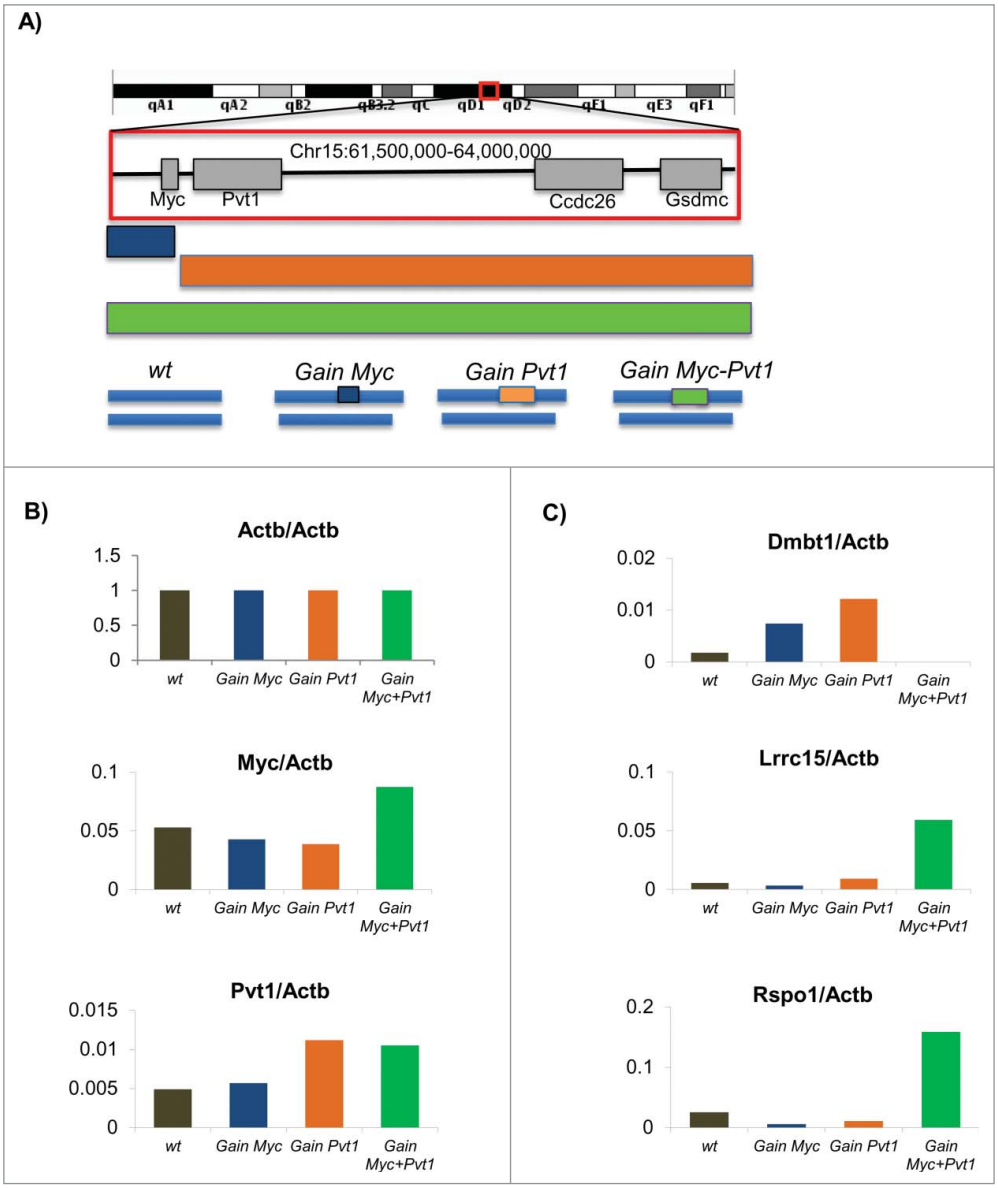

Figure 1. (A) Schematic of the engineered *MMTV-Neu* mouse model for the Gain (*Myc*), Gain (*Pvt1*) and Gain (*Myc-Pvt1*) animals from which mammary epithelium was obtained. Following RNA-SEQ analyses FPKM transcript abundance further normalized by the levels observed for Actb for (B) Actb, Myc and Pvt1 and (C) Dmbt1, Lrrc1, and Rspo1 are plotted using colors defined in the engineering schematic above.

signaling pathway.[10] *Rspo1* is involved in female specific differentiation.[11] Intriguingly, Rspo1 is required for normal epithelial morphogenesis during mammary gland development. Specifically, mammary tissue from Rspo1 null animals fail to exhibit ductal branching,[12] exactly the phenotype observed to be present in excess in the *Gain(Myc-Pvt1)* mammary tissue.

Increased RSPO1 expression in human breast cancer with high MYC and PVT1

We next examined the expression levels of *MYC, PVT1* and *RSPO1* in The Cancer Genome Atlas breast cancer RNA-SEQ dataset[13] to find whether *Myc-Pvt1* mediated increases in *Rspo1* levels are important to a subset of human tumors. We hypothesized that sorting the data based on *MYC* expression would show that high *MYC* levels are associated with high levels of *PVT1* and that a subset of these tumors would also show increases in *RSPO1*. The top quartile of the tumors sorted by MYC expression was compared with the bottom quartile of tumors by 2-group T-test (Fig. 2A). As expected, highly significant increases in MYC transcript were observed between tumors with high levels of *MYC* and low levels of *MYC* (P-val $< 10E-16$, average fold change $= 8.3$) (Fig. 2B). Confirmatory of a role for *MYC* and *PVT1* co-operation in cancer, tumors with high levels of *MYC* showed highly significant increases in PVT1 transcript (P-val $= 10E-11$, average fold change 2.3). *RSPO1* levels were also significantly increased in tumors with high levels of *MYC* compared to tumors with low levels of *MYC* (P-val $= 0.03$, average fold change 3.1). *ACTB* levels were not significantly different between the high MYC and low MYC group (P-val 0.55, average fold change 0.98).

Role of RSPO1 in sex specification and cancer

By looking at low-level copy number changes within the context of normal organismal development we increase the likelihood of observing relevant events early in tumor formation. In an attempt to catch precancerous cells in the

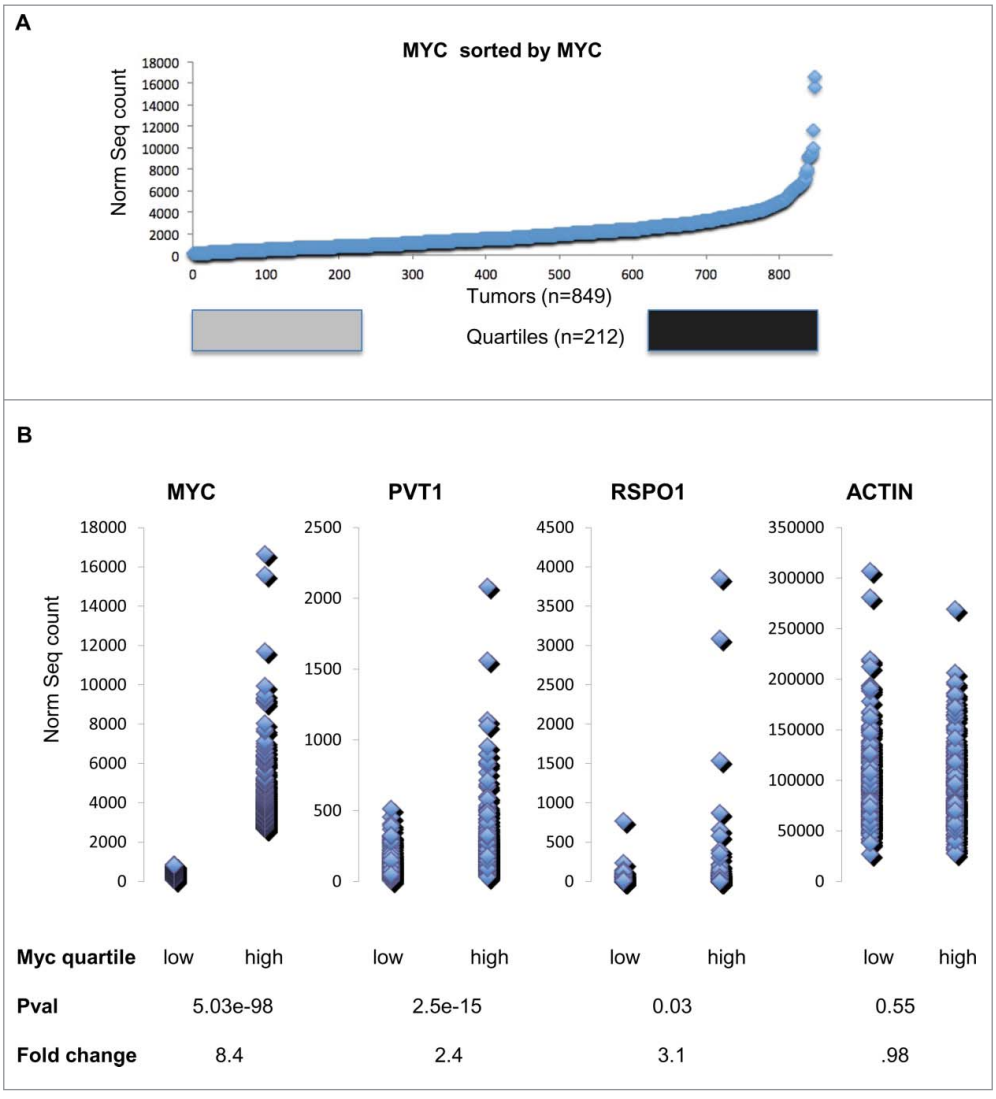

Figure 2. (A) Myc transcript abundance for 849 TCGA Breast cancer tumors sorted by increasing Myc transcript abundance. Tumors within the bottom Quartile are shown in gray and tumors within the top quartile are shown in black. (B) Dot plot comparison of bottom quartile of transcript values compares to top quartile transcript for Myc, Pvt1, Rspo1 and ActB. A two group T-test shows that the Pvt1 and Rspo1 are both significantly higher and show a large average fold change in the top quartile relative to the bottom quartile based on Myc transcript level. ActB is not significantly different by T-test and also by fold change.

early stages of tumorigenesis we utilized RNA sequencing to identify potential targets of supernumerary *MYC-PVT1* cooperation in mammary epithelial cells. Here we show that an extra copy of both *Myc* + *Pvt1* leads to increased levels of *Rspo1*, a crucial regulator of canonical β-catenin signaling required for female development. We further show that human tumors with high levels of *MYC* also show high levels *PVT1* and *RSPO1*.

Other members of the *RSPO* gene family have previously been implicated in cancer. Recurrent fusions *in RSPO2* have been observed in human colon cancer.[14] High RSPO1 levels have been associated with poor outcome in glioma patients.[15] It has also been found to be a prominent susceptibility locus in ovarian cancer.[16] Further *Rspo1* has been identified as common insertion site in murine forward genetic tumor screens and the T2/Onc insertions are oriented consistent with transcriptional activation of *Rspo1*.[17]

That a gene involved in sex determination would also play a role in cancer formation has precedent. A key transcriptional regulator of the male expression pattern is also involved in tumorigenesis. Deletion of *Dmrt1* leads to male to female sex reversal[18] and multiple variants in *DMRT1* have been associated with germ cell tumors.[19] Likewise, *RSPO1* mutations have been associated with female to male sex reversal and tumor formation.[20] In both mammary and testes tissue developmentally important mechanisms exist to allow cell division in mature tissues and these mechanisms appear to be misappropriated during the tumor formation processes. Therefore, sexual differentiation programs appear to have the potential to allow tumor promoting behavior, because they contain signal dependent proliferation programs for differentiated cell types.

The molecular mechanism of the *MYC - PVT1* synergy leading to the activation of *RSPO1* needs to be investigated in detail. We hypothesize that in addition to stabilization of *MYC* protein, *PVT1* may retarget the *MYC* complex modifying the transcriptional response in the presence of Estrogen. The concept that *MYC* mediated responses such as proliferation could be retargeted in a cell dependent fashion is attractive considering the general role that *MYC* plays in tumorigenesis as well as the wide variety of molecular mechanisms attributed to *MYC* increase which run the gamut of apoptosis to proliferation to induced pluripotency. Tissue specific MYC retargeting opens the door to tissue specific MYC inhibition.

In summary our current work provides a specific molecular rational for the precancerous phenotypes observed with the Gain *Myc-Pvt1* and expands the target list for pharmacological intervention downstream of MYC- PVT1 cooperation in both cancer and precancerous tissue. Further analyses of these biomarkers of precancerous state in "normal" human breast tissue may help to stratify populations at risk of developing breast cancer prior to the physical tumor diagnosis.

Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

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