Epigenetic changes in fibroblasts drive cancer metabolism and differentiation

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Abstract

Genomic changes that drive cancer initiation and progression contribute to the co-evolution of the adjacent stroma. The nature of the stromal reprogramming involves differential DNA methylation patterns and levels that change in response to the tumor and systemic therapeutic intervention. Epigenetic reprogramming in carcinoma-associated fibroblasts are robust biomarkers for cancer progression and have a transcriptional impact that support cancer epithelial progression in a paracrine manner. For prostate cancer, promoter hypermethylation and silencing of the RasGAP, RASAL3 resulted in the activation of Ras signaling in carcinoma-associated fibroblasts. Stromal Ras activity initiated a process of macropinocytosis that provided prostate cancer epithelia with abundant glutamine for metabolic conversion to fuel its proliferation and a signal to transdifferentiate into a neuroendocrine phenotype. This epigenetic oncogenic metabolic/signaling axis seemed to be further potentiated by androgen receptor signaling antagonists and contributed to therapeutic resistance. Intervention of stromal signaling may complement conventional therapies targeting the cancer cell.

Key Words

endocrine therapy resistance
prostate
neuroendocrine tumors

Chromatin modification in cancer: a brief overview

Cancer is a general term for a group of diseases that diverge with respect to its origin and is characterized by uncontrolled proliferation with the potential for metastatic progression (Hanahan & Weinberg 2000, Chaffer & Weinberg 2011, Hanahan & Weinberg 2011). Cell proliferation is normally tightly regulated at the gene level with dynamic chromatin modifications (Perino & Veenstra 2016). Chromatin structure is central for the regulation of gene expression either by organizing the structure of promoters and regulatory elements or by providing accessibility to transcription factor binding at regulatory elements (Tirosh & Barkai 2008). One of the prime epigenetic phenomena in cancer is suppression or downregulation of tumor suppressor genes through aberrant promoter methylation and deacetylation, often associated with condensing the chromatin structure and preventing transcription factor loading, resulting in gene silencing (Robertson 2001, Luczak & Jagodziński 2006). Conversely, the acetylation and demethylation of the gene-body can also result in gene silencing. The epigenetic activation of oncogenes on the other hand seem to be less associated with direct DNA or histone methylation/acetylation of the oncogenes themselves, but rather miRNAs that can indirectly regulate tumorigenic potential
The addition of a methyl group ($\text{CH}_3$) at fifth carbon position of the cytosine ring of DNA, termed, 5-methylcytosine (5mC), predominantly occurs in CpG-rich sequences. Somatic, non-stem cells, normally have hypomethylated CpG islands in promoter sequences (Moore et al. 2013). However, aberrant promoter hypermethylation of multiple tumor-suppressor genes is associated with the upregulation of DNA methyltransferases (DNMTs) in multiple cancer types (Jin & Robertson 2013, Moore et al. 2013). The DNMT family comprises four members which include DNMT1, DNMT3A, DNMT3B and DNMT3L. All members of the family possess inherent enzyme activity except DNMT3L (Jin & Robertson 2013). While DNMT1 functions during DNA replication to maintain the DNA methylation pattern from the parental DNA strand onto the newly synthesized daughter strand, DNMT3a and DNMT3b are responsible for establishing de novo methylation pattern to unmodified DNA (Okano et al. 1998, 1999, Riggs & Xiong 2004, Egger et al. 2006, Goll et al. 2006). Epigenetic cancer therapeutic targets DNA/histone methylation in order to reverse chromatin remodeling (Sproul & Meehan 2013). An feature of cancer cell is the reduced total global DNA methylation in the context of enriched DNA methylation at certain promoter CpG islands (Wu et al. 2018). Laird et al. showed that heterozygotic mice with null mutation of Dnmt1, when treated with specific inhibitors of DNA methylation, such as 5-aza-2’-deoxycytidime (5-aza-dC) significantly reduced tumor formation in Apc Min^{+/-} mice (Takebayashi et al. 2007). Additional studies with gene knockout analysis in mice have shown that, a Dnmt1 hypomorphic allele (causing partial loss of function) can suppress polypl formation and CpG island methylation (Eads et al. 2002). In particular, studies have demonstrated that DNMT1 overexpression correlates with colon tumors, compared to non-malignant adjacent stroma (Honeywell et al. 2018). DNA methylation marks also involve active demethylation of 5mC by oxidizing enzymes including the ten-eleven translocation (TET) enzymes (TET1, TET2, TET3) as well as associated histone proteins by demethylase KDM4A/JHDM2A. Interestingly, epigenetic regulation can itself be regulated by metabolic intermediates. For example, the TCA cycle metabolite α-ketoglutarate is an inducer of TET2 (Raffel et al. 2017). The subsequent downstream metabolites, succinate and fumarate, promoted histone demethylation by KDM4A/JHDM2A (Xiao et al. 2012). New findings on the relationship between chromatin modification and cancer metabolism provide new opportunities for epigenetic therapy.

**Epigenetic coevolution of stromal fibroblastic cells in response to tumorigenesis**

It is now established that carcinogenesis involves reciprocal interactions between cancer cells and components of the surrounding microenvironment consisting of extracellular matrix, fibroblasts, vasculature-associated endothelia and pericytes, as well as immune cells and occasionally adipose cells (Plava et al. 2019). Based on the pro-tumorigenic role these non-tumorigenic components have, tumor microenvironment-targeted interventions have attracted notable attention in cancer therapy (Dey 2011, Quail & Joyce 2017). Prominently, angiogenesis inhibitors have been practice-changing for a few cancer types, but interestingly had a lesser impact on cancer care than originally anticipated. Regulators of fibrosis have had limited efficacy. While immune therapy targeting T cell activation has taken cancer care by storm recently, thus far under 20% of melanoma and lung cancer patients demonstrate lasting benefit. Interestingly, there is a distinct change in the chromatin-accessible regions of exhausted T cells that is not alterable by immune checkpoint inhibition (Pauken et al. 2016, Sen et al. 2016). The understanding of the most abundant cell type of the solid tumor microenvironment, the fibroblasts, remains largely unknown. Not without controversy, cancer-associated fibroblasts (CAF), is considered not to be driven by genomic mutations (Hill et al. 2005, Li et al. 2007, Qu et al. 2008, Bianchi-Frias et al. 2016). However, the seminal finding by Cunha and colleagues that CAFs have the capacity to maintain its tumor-inductive capacity in the absence of the constant signals from cancer cells for a period of time, suggested an inherent ‘memory’ (Olumi et al. 1999, Hayward et al. 2001). As evidence, CAF can be isolated from patient tissues, cultured, and then transferred to mice with non-tumorigenic cells to develop a tumor. In the absence of mutations, the pro-tumorigenic phenotype of CAF is found to be driven by
epigenetic mechanisms associated with promoter DNA methylation (Dumont et al. 2008, Gascard & Tlsty 2016).

CAF s are the dominant cell type in tumor microenvironment, with both pro- and anti-tumorigenic capacity (Placencio et al. 2008, Kalluri 2016, LeBlu & Kalluri 2018). The net effect of paracrine signaling crosstalk between CAF s and the cancer epithelia provides avenues for disrupting pro-tumorigenic signaling (Wu et al. 2012). In contrast to normal tissue-associated fibroblasts (NAFs), the epigenetic programming in CAFs represents a durable change that is able to promote tumor growth (Fiori et al. 2019). The distinct contribution of TME epigenetic landscapes in tumorigenesis was first highlighted by Hu and colleagues (Hu et al. 2005) by developing a novel method – methylation-specific digital karyotyping tissue obtained from epithelial and stromal fibroblasts from normal breast and in situ and invasive breast carcinomas. This study highlighted that epigenetic landscape has a role in the maintenance of the abnormal microenvironment in breast cancer. In prostate cancer, pi-class glutathione S-transferase gene (GSTP1) promoter is methylated in >90% cases (Lee et al. 1994). This seminal study demonstrated distinct GSTP1 gene promoter methylation of the stromal cells in prostate cancer. Although the primary role of GSTP1 is in the detoxification of carcinogens (Allocati et al. 2018), it is not involved in the suppression of cancer cell growth and cannot be classified as a tumor suppressor gene (TSG); however, its aberrant silencing in CAFs may create a permissive microenvironment for tumorigenesis (Lee 2007). In agreement Rodriguez-Canales et al. demonstrated significant topographical differences and distinct area of stromal methylation of the stroma especially at the center of the tumor in the prostate using laser capture microdissection (Rodriguez-Canales et al. 2007). We have reported that the epigenetic silencing of the TGF-β type II receptor (Tgfb2) in prostatic CAF can be causative for GSTP1 promoter methylation, as the knockout of the Tgfb2 resulted in GSTP1 silencing in addition to a number of DNA damage repair genes (Banerjee et al. 2014). In addition, prostatic human CAF and mouse transgenic knockout of Tgfb2 demonstrated elevated DNA methyltransferases 1 (DNMT1) activity and histone H3 lysine 9 trimethylation (H3K9me3) associated with greater promoter methylation. Notably, restoring the expression of the epigenetically silenced genes in the CAF using 5-azacitidine led to reduced tumor progression (Banerjee et al. 2014). Promoter DNA and histone methylation can mediate a tumor permissive environment (El-Osta & Wolfe 2000, Rose & Klose 2014). A recent study showed that CAFs with a large number of H3K27me3 changes had greater tumor-promoting effects, associated with the secretion of the paracrine factor WNT5a (Maeda et al. 2019). The epigenetic landscape of PCa CAF has diagnostic and grading capacity of PCa (Gordetsky & Epstein 2016, Pidsley et al. 2018).

DNA methylation and histone modification studies in CAF

Recent advancement in ‘omics’ technologies have allowed for genome-wide profiling of genome-scale DNA methylation both at a single-nucleotide and at a single-cell resolution (Lo & Zhou 2018). These methylation techniques are primarily based on the concept that treatment of sodium bisulfite on DNA leads to the conversion of nonmethylated cytosines to uracil whilst maintaining 5-methylcytosine (5mC) unchanged (commonly called as protected region) (Clark et al. 1994). Bisulphite conversion is still considered to be the ‘gold standard’ to detect DNA methylation patterns. In addition, alternative methylcytosine-specific enrichment technologies, such as methylated DNA immunoprecipitation (MeDIP) and methyl-CpG-binding technologies are region-based approaches in whole genomes, therefore, do not deliver the detail of DNA methylation patterns (Bock et al. 2010). Incorporation of next-generation sequencing methods with bisulfite conversion is the basis for reduced representation (RRBS) or whole genome (WGBS) data to identify genome-wide CpG coverage (Harris et al. 2010). We performed first application of RRBS technology in analyzing DNA methylation pattern in fibroblasts (Mishra et al. 2018). Comparing the DNA methylome analysis of prostatic NAF and CAF, we recognized genes that had reported roles in tumor progression, suppression, and metastasis (Table 1). There were 18 tumor-promoting, 11 suppressing, 2 metastasis regulatory gene promoters’ hypermethylated in the prostatic CAFs. Heat maps of the genes suggest critical novel biomarkers for prostate cancer (Fig. 1). The rational for focusing on known tumor regulators in the non-transformed fibroblastic cells is based on significant evidence that such genes in fibroblasts have distinct paracrine effects on associated epithelia. Indeed, the forced expression of two oncogenic events are required to transform embryonic fibroblasts (Land et al. 1983). However, the effects on adjacent epithelia only seem to require a single such hit. For example, the loss of tumor suppressors, such as TGFB2 or phosphatase and tensin homolog (PTEN) in prostate and breast fibroblasts, respectively, has been associated with greater promoter DNA methylation.
| Name       | Description                                      | Biological effect                                                                 | Reference                  |
|------------|--------------------------------------------------|------------------------------------------------------------------------------------|----------------------------|
| Oncogene   | Mesenteric estrogen-dependent adipogenesis       | Observed in almost all cases of papillary thyroid carcinomas. High expression was   | (Song et al. 2019)         |
| MEDAG      |                                                  | correlated with metastasis and poor disease-free survival.                         |                            |
| ALX1       | Aristaless-like homeobox1                        | Induces EMT and cell invasion in ovarian cancer cells by promoting Snail           | (Yuan et al. 2013)         |
| CACNA1C    | Calcium voltage-gated channel subunit alpha1 C   | Expression was directly regulated by miR-363 whose high expression is associated   | (Zhang et al. 2019)        |
| GPT2       | Glutamic pyruvate transaminase GPT2              | Promotes tumorigenesis and stemness of breast cancer cells by activating the Shh    | (Cao et al. 2017)          |
| HSPA2      | Heat shock-related 70-kDa protein 2              | Overexpression is correlated with tumor angiogenesis and poor prognosis in         | (Zhai et al. 2017)         |
| PVRL4      | Poliovirus-receptor-like 4                       | pancreatic carcinoma.                                                             | (Pavlova et al. 2013)      |
| LAMA3      | Laminin alpha 3                                  | The analysis identified a splice variant known to be involved in tumor cell         | (Moller-Levet et al. 2009) |
| NOS2       | Nitric oxide synthase                            | Its expression was associated with brain metastases in mouse models of orthotopic   | (Heinecke et al. 2014)     |
| FOXD2-AS1  | FOXD2 adjacent opposite strand RNA1              | Promoted the progression of colorectal cancer by regulating EMT and Notch          | (Yang et al. 2017)         |
| SFRP4      | Secreted frizzled-related protein 4 (SFRP4)      | Elevated gene expression is associated with high grade disease and recurrent prostate | (Sandsmark et al. 2017)    |
| SH3RF2     | SH3-domain-containing RING finger protein        | Regulates p21-activated kinase 4 (PAK4) protein stability. Ectopic expression limit  | (Kim et al. 2014)          |
| CD74       | Cluster of Differentiation 93                    | In several forms of cancer, CD74 is up-regulated and associated with enhanced       | (Schroder 2016)            |
|            |                                                  | proliferation and metastatic potential                                           |                            |
| COBL       | cordon-bleu WH2 repeat protein                   | It is involved in the cancer cell morphogenesis, implicated in the acquisition of   | (Lopes et al. 2016,         |
|            |                                                  | the neuron-like cell shape observed in neuroendocrine prostate cancer.            | Takayama et al. 2018)      |
| NAV1       | Neuron navigator 1                               | Expressed in brain astrocytoma, its expression was positively correlated with the    | (Xing et al. 2014)         |
| B3GNT1     | β-1,3-N-acetylglucosaminyltransferase 1          | degree of malignancy                                                             | (Buysse et al. 2013)       |
| CD93       | Cluster of Differentiation 93                    | A key regulator of glioma angiogenesis, acting via cytoskeletal rearrangements      | (Langenkamp et al. 2015)   |

(Continued)
| Name          | Description                                      | Biological effect                                                                                                                                                                                                 | Reference                      |
|---------------|--------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| NTRK1         | Neurotrophic receptor tyrosine kinase 1          | Tumor samples from 3 of 91 patients with lung cancer (3.3%) without known oncogenic alterations assayed by next-generation sequencing or fluorescence in situ hybridization demonstrated evidence of NTRK1 gene fusions | (Vaishnavi et al. 2013)        |
| SIX2          | SIX homeobox 2                                   | Transcription factor involved in organ development and breast cancer stem cells through the positive regulation of SOX2                                                                                             | (Wang et al. 2014, Oliphant et al. 2019) |
| Tumor suppressor |                                                                 |                                                                                           |                                |
| FES           | c-fes protein-tyrosine kinase                    | Expression downregulated in colon tumors. Restoration of expression suppressed their colon cancer growth in soft agar.                                                                                          | (Delfino et al. 2006)          |
| LSP1          | Lymphocyte-specific protein 1                    | Inhibits the growth of hepatocellular carcinoma by suppressing ERK1/2 phosphorylation. Patients with high LSP1 expression had significantly better overall survival.                                      | (Zhang et al. 2016)            |
| LIMCH1        | Lim and calponin-homology domains 1              | Potentiates actin stress fiber assembly and stabilizes focal adhesions to negatively regulate cell spreading and migration                                                                                       | (Lin et al. 2017)              |
| CDYL          | Chromodomain on y-like                           | CDYL bridges REST and histone methyltransferases for gene repression and suppression of cellular transformation. Loss of heterozygosity associated with cervical cancer transformation. | (Mulligan et al. 2008)         |
| CCDC68        | Coiled-coil domain containing 68                 | Allows for centriol anchoring to microtubules in interphase cells. Directly associated with pancreatic cancer proliferation.                                                                                     | (Radulovich et al. 2015)       |
| ISYNA1        | Inositol 3-phosphate synthase (ISYNA1)           | Ectopic ISYNA1 expression increased myo-inositol levels in the cells and suppressed tumor cell growth.                                                                                                         | (Koguchi et al. 2016)          |
| LZTS3         | Leucine zipper tumor suppressor family member 3  | In silico characterization of LZTS3 identified its potential tumor suppressor.                                                                                                                                     | (Teufel et al. 2005)           |
| ING3          | Inhibitor of growth                              | Can activate p53 trans-activated promoters, including promoters of p21/waf1 and Bax. Overexpression can inhibit cell growth and induce apoptosis in head and neck cancers.                                              | (Gou et al. 2014)              |
| TBX4          | T-box transcription factor Tbx4                  | Reduced expression suggests a worse prognosis for pancreatic cancer patients.                                                                                                                                     | (Zong et al. 2011)             |
| RPL23A        | Ribosomal protein L23A gene                      | A component of the 60S ribosomal subunit exhibits anti-cancer function on the Hep-2 cells.                                                                                                                                 | (Sun et al. 2012)              |
| HOXA5         | Homeobox A5                                      | Loss of expression occurs frequently in breast cancer and correlates with higher pathological grade and poorer disease outcome.                                                                                   | (Teo et al. 2016)              |
| Metastasis    | ESRP1 Epithelial splicing regulatory protein 1   | Drives a switch from mesenchymal to epithelial phenotype characterized by reduced cell migration of ovarian cancer                                                                                               | (Jeong et al. 2017)            |
|               | ANXA2 Annexin A2                                 | High-affinity binding for Ca and phospholipids like other annexin family members. Implicated in multiple cancer types to greater metastasis and poor prognosis.                                                     | (Christensen et al. 2018, Li et al. 2019) |
with breast and prostate cancer mouse models (Bhowmick et al. 2004, Cheng et al. 2005, Trimboli et al. 2009). In parallel, oncogene expression of cyclin D1 (CCND1) and CMYC in the CAF has been reported to promote tumorigenicity in PCa models (He et al. 2007, Valencia et al. 2014, Minciacchi et al. 2017). In fact, gastric cancer-associated stromal methylation signature was found to be a determinant of epithelial tumor stage (Jiang et al. 2008). Methylation-sensitive SNP array analysis (MSNP) was used to compare DNA methylation in NAF and CAF cells. Fewer genes were found to have promoter hypermethylation in CAFs compared to NAF (Jiang et al. 2008). Aberrant DNA methylation pattern in CAFs that affected TGF-β signaling was found to be prognostic for non-small-cell lung cancer patients (Vizoso et al. 2015). CAF in pancreatic ductal adenocarcinoma, associated with extensive connective tissue deposition, had a distinct methylation landscape that promote malignant growth and progression. Suppressor of cytokine signaling (SOCS) family gene, SOCS1 was identified as a prominent gene frequently methylated in pancreatic CAFs (Xiao et al. 2016). Conversely, the ADAM12 gene promoter was hypomethylated in pancreatic CAFs (Yu et al. 2012). Together, these data demonstrate stromal DNA methylation status can impact cancer progression.

In a noteworthy study, Albrengues et al. demonstrated that an epigenetic switch involving the leukemia-inducible factor (LIF), a proinflammatory cytokine of IL-6 class secreted by cancer cells, reprograms human head and neck CAF into a state that supported cancer cell invasion via extracellular matrix (ECM) remodeling (Albrengues et al. 2015). They further showed that DNMT3B methylated CpG sites of the SHP-1 phosphatase promoter to downregulate SHP-1 expression, resulting in constitutive phosphorylation of JAK1. Thereafter JAK1/STAT3 signaling was sustained by maintenance methylation enzyme, DNMT1. This study provided a unique link of histone modification and DNA methylation in fibroblasts. The authors observed that DNMT inhibitor, 5-AzaDC, restored the expression of SHP-1, thereby decreasing JAK1/STAT3 activation, and tumor-inductive properties of the fibroblasts. All together, these studies demonstrated crucial role of DNA methylation activity of the tumor microenvironment provided sustained head and neck cancer proinvasive activity. Histone methylation is also crucial for fibroblast activation. Accordingly, Tyan et al. reported that the loss of EZH2 (enhancer of zeste homolog 2) caused promoter-associated histone H3K27 methylation at the ADAMTS1 gene (ADAM metallopeptidase with thrombospondin type 1 motif), accounting for its enhanced expression (Tyan et al. 2012). These studies supported the role of epigenetic modification in breast stromal fibroblasts in conferring a tumor-inductive phenotype. Apart from histone modification, non-histone chromatin remodeling gene, Hmga2 (High-mobility group AT-hook 2) has been identified as an epigenetic regulator in prostatic fibroblasts. Stromal-specific overexpression of Hmga2 in mouse fibroblasts was sufficient for the induction of multifocal prostatic intraepithelial neoplasia in adjacent prostatic epithelia (Zong et al. 2012). More research is needed to understand the underpinning mechanisms for the emergence of the stable CAF phenotype. Figure 2 illustrates general epigenetic changes involved in fibroblast which alter cancer epithelial communications and proliferations.

**Epigenetic silencing of RasGAPs: alternative route to Ras signaling activation in cancer**

Altered Ras signaling has achieved notoriety in contributing to tumorigenesis (Fernandez-Medarde & Santos 2011). More than 30% of all human neoplasms...
R679
R Mishra et al.
TME based epigenetic targets in cancer

26:12
R769

harbor an oncogenic form of Ras proteins, made up of a small family of three closely related proteins (K-Ras, H-Ras, or N-Ras) (Adjei 2001, Canevari et al. 2002). As GTPases, Ras proteins oscillate between an active GTP-bound and guanosine diphosphate (GDP)-bound inactive state. The RasGAP family of proteins inactivate Ras signaling by binding Ras and catalyzing Ras-GTP hydrolysis to Ras-GDP (King et al. 2013, Simanshu et al. 2017, Scheffzek & Shivalingaiah 2019). The silencing of the RasGAP genes by promoter methylation results in the activation of RAS signaling and promote primary tumor development (Fernandez-Medarde & Santos 2011, Simanshu et al. 2017). In addition, the inactivation of the RasGAP, RASAL1, in fibroblasts can contribute to renal and cardiac fibrosis (Bechtel et al. 2010, Xu et al. 2015). There are 14 RasGAP genes identified in the human genome (Bernards 2003). We performed Oncomine analysis to investigate the differences in the mRNA levels of different RasGAPs genes, between tumor and normal tissues in multiple cancer types (Fig. 3). The epigenetic regulation of RasGAP proteins that contribute to activation of Ras signaling and its implication in tumorigenesis is further discussed below.

DAB2IP is one of most well-studied RasGAPs in cancers, also known as AIP1 (ASK1-interacting protein). Several studies reported DAB2IP gene regulation through aberrant methylation in prostate, breast, lung, liver and gastrointestinal cancers (Chen et al. 2003, Dote et al. 2004, 2005, Yano et al. 2005). A DNA methylation-based study conducted in renal cell carcinoma identified DAB2IP promoter methylation as a practical prognostic biomarker. The CpG methylation biomarker is located upstream of the transcription start site of DAB2IP (DAB2IP CpG1). Pyrosequencing quantitative methylation assay of over 550 patient paraffin renal cancer tissue sections was used to establish a correlation between DAB2IP CpG1 methylation and overall survival (Wang et al. 2016). Similarly, DAB2IP promoter methylation and expression downregulation were identified to be associated with breast cancer lymph node metastasis (Dote et al. 2004). The restoration of DAB2IP expression by 5-acetazolamide-2-cytosine deoxyriboside (5azaDC, DNA demethylating agent) supported the epigenetic regulation of breast cancer progression (Dote et al. 2004). Methylation of DAB2IP exon 3 was associated with histone H3 di- and trimethyl H3-Lys27 (H3K27me2 and H3K27me3), a site known to be modified by EZH2 and recruitment of polycomb repressive complex 2 and histone deacetylases (Chen et al. 2003, Smits et al. 2012). The established tumor-suppressive role of DAB2IP has been extended to its role in angiogenesis inhibition and chemo/radiation sensitization, to reveal some Ras-independent effects of this RasGAP.

RASAL1 has been identified as a tumor suppressor, frequently silenced by promoter hypermethylation in numerous cancer types. For example, screening of 13 RasGAPs in 12 human thyroid cancer cell lines revealed epigenetic silencing of RASAL1 (Liu et al. 2013). Notably, treating these cell lines with 5azaDC restored RASAL1 expression. In another example, promoter hypermethylation of RASAL1 was found in colorectal cancers, interestingly frequently also associated with
K-Ras mutational activation (Ohta et al. 2009). Ectopic expression of RASAL1 or using a DNA methylation inhibitor was found to reduce Ras signaling and colon cancer progression (Liu et al. 2005, Ohta et al. 2009). Likewise, RASAL1 promoter DNA hypermethylation in gastric cancer tumor tissues were greater than that in paired adjacent non-tumor tissues (Chen et al. 2013). Apart from DNA methyltransferases, there are also histone-modifying enzymes, which can play a role in the regulation of RASAL1. Brigette et al. revealed that treatment with histone deacetylase inhibitor (HDACi), belinostat (PXD101), led to a modest restoration of RASAL1 expression in HepG2 and Hep3b cell lines (Ma et al. 2010). For these diverse cancer types with RASAL1 epigenetic silencing, often associated with Ras-driven carcinogenesis, the added loss of the suppressor potentially super-activates the Ras signaling axis. In the same models, however, the restoration of RASAL1 expression was found to negate some of the effects of the endogenous Ras-activating mutations or amplification. Thus, epigenetically regulated RasGAP activity can be considered to be dominant over such genomic alterations of the Ras gene.

Epigenetic silencing of RASAL2 has demonstrated that it can function as a tumor and metastasis suppressor, in breast cancer, hepatocellular carcinoma, colorectal cancer (Jia et al. 2017), nasopharyngeal carcinoma, lung cancer, and ovarian cancer (McLaughlin et al. 2013, Feng et al. 2014, Huang et al. 2014, Li & Li 2014, Stefanska et al. 2014, Wang et al. 2015, Yan et al. 2016, Olsen et al. 2017). Notably, promoter hypermethylation of RASAL2 and DAB2IP was identified in aggressive luminal B breast cancer (Olsen et al. 2017). Performing gain-of-function and loss-of-function studies, Hui et al. demonstrated that formation of new blood vessels was suppressed by RASAL2 via VEGFA downregulation in renal cell carcinoma metastasis (Hui et al. 2018). Further, the epigenetic silencing of RASAL2 was negatively correlated with the overall survival of renal cell carcinoma patients (Hui et al. 2018).

Unlike the other two RASAL family members, RASAL3 has not been considered a tumor suppressor in the

Figure 3
The expression levels of human RASAL1, RASAL2, RASAL3 and DAB2IP are profiled across multiple cancer types, compared to normal tissue by Oncomine. The gene expression level differences between cancer and normal tissue are illustrated. The number of datasets in which statistically significant mRNA overexpression or under-expression was observed is indicated in red or blue boxes, respectively. The color intensity corresponds to the gene rank and magnitude of expression differences with a statistically significant threshold.
Enhanced macropinocytosis provide metabolic flexibility for tumor cells

In Ras-driven cancers like pancreatic and glioblastoma, a process of uptake of albumin and other macromolecules from its surroundings, termed macropinocytosis occurs (Commissio et al. 2013, Muller-Greven et al. 2017). Subsequent albumin translocation to lysosomes generates amino acids. And as albumin is rich in glutamine, an outcome of macropinocytosis is glutamine efflux. For pancreatic cancer, the further metabolism of glutamine serves as a means of fueling cancer progression. However, we found that prostatic CAFs do not seem to metabolize the glutamine further (Mishra et al. 2018). Rather, glutamine gets secreted for its uptake by adjacent cancer epithelia, where it is metabolized to glutamate and enters the TCA cycle via α-ketoglutarate. A key hurdle for cancer cells is to fulfill rising energy demand for the growing biomass in often nutrient-depleted conditions (DeBerardinis & Chandel 2016). In order to meet energy/biosynthetic demand, tumors have evolved tremendous capacity to reprogram pathways triggering nutrient acquisition. Metabolic reprogramming is recognized as one of the hallmarks of cancer (Hanahan & Weinberg 2011) and explored as therapeutic targets (Altman et al. 2016, DeBerardinis & Chandel 2016, Cluntun et al. 2017). We demonstrated that the uptake of glutamine by amino acid transporter (SLC1A5), as well the metabolism of glutamine to glutamate by glutaminase (GLS) was upregulated in the cancer epithelia in response to elevated concentrations of glutamine in the media (Mishra et al. 2018). Macropinocytosis is one of the important strategies that cancer cells use as an alternative nutrient acquisition pathway (Commissio et al. 2013, Zwartkruis & Burgering 2013, Nakase et al. 2015, Wang et al. 2018). While the first microscopic observations of macropinocytosis in malignant cells was discovered in 1930s, its mechanistic understanding occurred in the last few years. The uptake of macromolecules through a specialized process of plasma membrane ruffling for the formation of endocytic macropinosomes that fuse into lysosomes is now an established process for anabolic metabolism for cancer cells (Recouvreux & Commissio 2017, Wang et al. 2018). Apart from oncogenic Ras activation, phosphatidylinositols 3-kinase (PI3-kinase) and phosphatase and tensin homolog (PTEN) mutations found in cancer (Chalhoub & Baker 2009) may also potentiate macropinocytosis as an adaptation to limiting nutrient availability (DeBerardinis & Chandel 2016, Cluntun et al. 2017, Recouvreux & Comissio 2017). However, in the case of PCa, the stromal co-evolution with the cancer epithelia involve epigenetic imprinting associated with RASAL3 silencing. This particular stromal reprogramming supports cancer progression via the induction of fibroblastic activation and secretion of glutamine.

The role of glutamine as a conditionally essential amino acid for cancer cells is well documented as a critical metabolite for nucleotide biosynthesis and anaplerosis. In addition, we found that incubation of PCa cells with glutamine resulted in the expression of neuroendocrine markers. We demonstrated that the uptake and metabolism of glutamine by SLC1A5 and GLS, respectively, was critical to the differentiation of prostate adenocarcinoma to the neuroendocrine phenotype. Neuroendocrine prostate cancer (NEPC) cells loose
granular structure and tend to have a small cell-like morphology characterized by the varying levels of expression of neuronal markers, including chromogranin A (CGA), synaptophysin (SYP), neurospecific enolase (NSE), and more recently T-Box brachury (Blaschko et al. 1967, Schmechel et al. 1978, Wiedenmann et al. 1986, Pinto et al. 2016). De novo NEPC is one of the rarest form (<1%) of the disease (Gupta & Gupta 2017). However, in response to AR signaling inhibition and/or androgen deprivation therapy, transdifferentiation to NEPC can support rapid disease progression with universally poor outcome, with an overall 5-year survival rate of 12.6% (Beltran et al. 2014, Yadav et al. 2016). Importantly, the transdifferentiated NEPC does not necessarily exhibit all the characteristics of de novo NEPC (Beltran et al. 2012, 2014). For example, transdifferentiated NEPC often maintains responsiveness to androgens despite its resistance to AR signaling inhibitors. While its incidence in primary prostate cancers is exceedingly low, in metastatic castrate-resistant prostate cancers (CRPCs), its percentage goes up to 25–30% (Gupta & Gupta 2017). Paracrine glutamine signaling is a mechanism by which AR signaling inhibitors potentiate this phenotype. We validated this finding in PCa patients that were on androgen receptor signaling inhibitors and found that those patients that developed therapeutic resistance had significantly higher blood glutamine levels compared to those who remained sensitive to hormone therapy (Mishra et al. 2018).

### Therapeutic interventions in response to stromal co-evolution

There is a need for better understanding of NEPC with the approval of more effective inhibitors of AR signaling (i.e. enzalutamide, apalutamide, darolutamide) for advanced PCa. Genomic characterization of transdifferentiated NEPC phenotypic tumors revealed recurrent amplifications of MYCN and AURKA as well as lesions of RB1 and TP53 (Beltran et al. 2011, Tan et al. 2014). For example, MYCN mutations are found in 40% of NEPCs, but only observed in 5% of all other PCa (Beltran et al. 2011). However, expression of other recognized NEPC markers, CHGA, SYP, NCAM1, and ENO2, was heterogeneous. The role of biomarkers not only serve to characterize the tumor type, but may provide a clue as to an effective intervention. Aurora kinase (AURK) was a specifically targeted kinase for cancers driven by MYCN, such as NEPC, neuroblastoma, and hepatocellular carcinoma with significant efficacy in mouse models (Otto et al. 2009, Dauch et al. 2016, Lee et al. 2016). Since AURK was found to bind and stabilization of MYCN (Otto et al. 2009), its inhibition resulted in MYCN degradation and reduction in tumor volume in a model of NEPC (Lee et al. 2016). A subsequent phase II clinical trial for NEPC patients with a AURK inhibitor, alisertib, unfortunately did not meet its primary endpoint, but the subset of patients that exhibited elevated MYCN and AURK were found to gain significant clinical benefit (Beltran et al. 2019).

MYC amplification can contribute to the regulation of glutamine metabolism in prostate cancer. Cancers with MYC amplification exhibit elevated expression of amino acid transporters SLC1A5 and SLC38A5, as well as glutamine-metabolizing enzyme, GLS. Glutamine addiction of cancer cells can be exploited through the inhibition of amino acid transporters or inhibitors of glutamine metabolism. However, non-cancer cells are generally non-vulnerable to such glutamine deprivation (Chen & Cui 2015, Altman et al. 2016, Still & Yuneva 2017).
Understanding of compensatory pathways of glutamine metabolism may improve the efficacy of cancer treatments. A sensor for abundant ATP includes the inhibition of AMP kinase, a known blocker of mTOR signaling. Thus, glutamine metabolism causes inhibition of the inhibitor of mTOR, resulting in mTOR activation and its downstream transcription factor FOXM1 in potentiating the expression of a number of NEPC-associated genes (Mishra et al. 2018). FOXM1 is a known master regulator of cancer metastasis, the expression of multiple stem cell genes, as well as MYCN and AURK (Raychaudhuri & Park 2011). Blocking GLS with BPTES (bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide) inhibited NEPC transdifferentiation (Mishra et al. 2018) (Fig. 4). CB-839 is a glutaminase inhibitor that is shown to be safe in phase I clinical trials and considerably more potent than BPTES. Blocking glutamine uptake by using GPN (i-γ-glutamyl-p-nitroanilide), a SLC1A5 inhibitor was effective in reducing tumor growth in the context of a commonly administered androgen receptor signaling inhibitor, enzalutamide (Mishra et al. 2018). As metastatic castrate-resistant prostate tumors have elevated available glutamine in circulation and its uptake can potentiate resistance to current AR signaling inhibition, a richer understanding of this pathway would contribute to better PCa treatment strategies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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