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Johng S. Rhim
Anatoly Dritschilo
Richard Kremer *Editors*

Human Cell Transformation

Advances in Cell Models for the Study of Cancer and Aging

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Preface

The role of carcinogenic agents in the development of human cancer is defined using a variety of human cells as experimental model systems. Therefore, the study of human cell transformation in culture by carcinogenic agents is of particular importance for understanding the cellular and molecular mechanisms underlying human carcinogenesis. A conference on "Human cell transformation: Advances in cell models for the study of cancer and aging" was held at the McGill University Health Center, Montreal, Canada, on June 13 and 14, 2018. The aims of the conference were to present the state-of-the-art in the transformation of human cells in culture, as well as to provide insight into the molecular and cellular changes involved in the conversion of normal cells to a neoplastic state of growth.

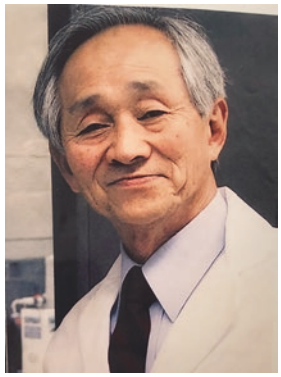
The conference encompassed the most recent development in human cell transformation including selected in vitro models, tumor xenografts, and transgenic preclinical models. The meeting provided a unique forum for the exchange of information in the important research field and ideas to promote scientific collaboration. Trainees had a unique opportunity to mingle with renowned scientists and presented their work at the poster session.

The following topics related to the theme of the conference.

1. Mechanisms of tumor progression
2. Prostate cancer progression
3. Skeletal dysregulation of cancer spread to the skeleton
4. Targeting the tumor and its microenvironment: new paradigms for cancer treatment

The conference was organized by johng S. Rhim, Anatoly Dritschilo, and Richard Kremer. There were 23 speakers, 23 poster presentations, and about 100 participants. The conference was well received and was perhaps the latest one devoted solely to the subject of human cell transformation in vitro. It is our privilege to have an opportunity to edit these proceedings and also on behalf of all contributors to thank everyone who have helped us produce this book.

Bethesda, MD, USA — Johng S. Rhim
Washington, DC, USA — Anatoly Dritschilo
Montréal, QC, Canada — Richard Kremer



Johng S. Rhim

Anatoly Dritschilo

Richard Kremer

Group speakers photograph of the conference of human cell transformation: Advances in cell models for the study of cancer and aging held on McGill University faculty club,Montreal, Canada on June 13-14, 2018.

Front row left to right. Back row left to right.

Front row left to right. Back row left to right.

Chronology of the Human Cell Transformation Meeting

1991 *Neoplastic transformation in human cell culture: mechanisms of carcinogenesis*. Georgetown University Medical Center, Washington, DC, USA. Organized by J.S. Rhim and A. Dritschilo.

1993 *International symposium on molecular mechanisms of radiation and chemical-induced cell Transformation*. Mackinac Island, MI, USA. Organized by K. Chadwick, A. Karaoglou, M. Frazin, V.M. Mahr, and J.J. McCormick.

1995 *Neoplastic transformation in human cell culture systems in cultures: mechanisms of Carcinogenesis*. Chicago, IL, USA. Organized by R.R. Weischenbaum, J.S. Rhim, A. Dritschilo, T.C. Yang, and J.C. Barrett.

1999 *In vitro transformation*. Cork, Ireland. A satellite meeting of the 11th international Congress of Radiation Research, Dublin, Ireland. Organized by J.S. Rhim and C. Mothersill.

2002 *Human cell culture 2002*, Oxford, UK. Joint meeting of European Tissue Culture Society, Scandinavian Society for Cell Toxicology and British Prostate Group, Oxford, UK. Organized by N.J. Maitland et al.

2010 *Human cell transformation: role of stem cells and the microenvironment*. McGill University Health Center, Montreal, QC, Canada. Organized by J.S. Rhim and Richard Kremer.

2018 *Human cell transformation: Advances in cell models for the study of cancer and aging*. McGill University Health Center, Montreal, QC, Canada. Organized by J.S. Rhim, A. Dritschilo and R. Kremer.

2019 Human cell transformation conference will be held at York, UK. Organized by N.J. Maitland, J.S. Rhim and F. Frame.

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Contents

Part I

Mechanisms of Tumor Progression

Mechanisms Underlying Metastatic Pancreatic Cancer

Jason R. Pitarresi and Anil K. Rustgi

Abstract

Pancreatic ductal adenocarcinoma is an overwhelming fatal disease that often presents with overt metastases and ultimately causes the majority of cancer-associated deaths. The mechanisms underlying the metastatic cascade are complex, and research in recent years has begun to provide insights into the underlying drivers of this phenomenon. It has become clear that cancer cells, in particular pancreatic cancer cells, possess properties of plasticity involving bidirectional transition between epithelial and mesenchymal identities. Furthermore, recent work has begun to establish that there are distinct hybrid states between purely epithelial and purely mesenchymal states that cancer cells may reside, in order to thrive at different stages of carcinogenesis. We discuss how this plasticity is important for different phases of the metastatic cascade, from delamination to colonization, and how different epithelial–mesenchymal states may affect metastatic organotropism. In this review, we summarize the current understanding of pancreatic cancer cell plasticity and metastasis, and highlight current model systems that can be used to study these phenomena.

Keywords

Pancreatic cancer · Epithelial-mesenchymal transition (EMT) · Cellular plasticity · Metastasis · Metastatic organotropism

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PDAC Metastasis and Outcomes

Pancreatic ductal adenocarcinoma (PDAC) is a major health issue in the USA, accounting for over 55,000 new cases and 44,000 deaths annually [1]. With fewer than 7% of patients surviving beyond 5 years and a projected increase in PDAC-associated deaths, PDAC will become the second leading cause of cancer deaths by 2020 [2]. Despite a significant emphasis being placed on improving early detection methods in PDAC patients, the majority of patients present with metastatic disease. The nearly universal mortality rate observed in PDAC is likely due to this unchecked metastatic potential.

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This dismal prognosis has not improved significantly for decades, likely due to the recently observed phenomenon that pancreatic cancer tumor cells are able to metastasize very early on during neoplastic transformation, before frank carcinoma is observed [3]. This observation, gleaned from genetically engineered mouse models (GEMMs) of PDAC, has rejuvenated the field of metastasis biology and provides an ideal model system for biologists to study the molecular underpinnings driving the metastatic cascade in this disease. The prevailing view in the field is that metastasis is facilitated through a process called epithelial-to-mesenchymal transition (EMT), where tumor cells lose their epithelial cell identity and begin to gain mesenchymal characteristics. In this chapter, we will discuss the role of epithelial cell plasticity in pancreatic cancer metastasis and introduce new paradigms thought to drive the metastatic cascade in this disease.

Models of Metastasis

Genetically Engineered Mouse Models of Metastatic PDAC

A landmark genetic mouse model of pancreatic cancer has become a mainstay in the field and has been a vital tool to shape our understanding of the molecular pathogenesis of PDAC [4]. Hingorani and colleagues looked to mimic the genetic mutations in human PDAC patients, which are dominated by activating mutations in *KRAS* codon 12, 13, or 61 (up to 95% of patients) and *TRP53* gain-of-function mutations (approximately 70% of patients) [5]. The resulting KPC mice, with inducible endogenous expression of mutant $\boldsymbol{K}ras^{G12D}$ and mutant $Tr\boldsymbol{p}53^{R172H}$ driven by the pancreatic epithelial cell-specific *Pdx1-**C**re* transgene, developed invasive and metastatic PDAC that mirrors the human disease. These mice succumb to disease with a median survival of approximately 5 months, at which point metastasis to the liver and lung is evident. KPC mice have re-shaped the field and revolutionized our understanding of PDAC biology, including seminal studies that have utilized the KPC mouse to establish roles for the TGF-ß pathway [6–8], Hedgehog signaling [9, 10], *Ink4a/Arf* [11, 12], *Brca1/2* [13–15], and other genes in PDAC tumorigenesis and progression. This now nearly ubiquitous model has provided an ideal model system to study the effect that genetic knockout of countless genes has on primary tumor formation and disease progression.

This model was expanded upon by Andrew Rhim in the laboratory of Ben Stanger through the introduction a Cre-inducible YFP lineage label driven by the same *Pdx1-Cre* transgene used to activate mutant $Kras^{G12D}$ and mutant $Trp53^{R172H}$ [3]. The subsequently named KCPY mice were used to show that dissemination occurs prior to frank malignancy and is driven by an underlying activation of an EMT program within the tumor cells. This model allows one to trace elegantly the metastatic process in pancreatic cancer, and is an invaluable resource in the field of metastasis biology. In particular, these mice grant us the ability to study of all stages of the metastatic cascade in vivo, from early invasion and growth into the surrounding tissue, to intravasation into the vasculature, travel through circulation, extravasation at the metastatic site, and colonization of the secondary tumor.

Orthotopic Models of Pancreatic Cancer Metastasis

The field has also adopted an orthotopic model of pancreatic cancer to quickly address the roles that a given gene may have during tumorigenesis and metastasis. Orthotopic injection of pancreatic tumor cells directly into the pancreas is an elegant approach that has been utilized for many years to efficiently test the effect of genetic alterations in PDAC tumor cells. Direct injection of KPC tumor cell cultures into the pancreas of syngeneic mice (or nude mice if cells are derived from a mixed background) yields primary tumors within days and metastatic lesions within weeks (protocol for orthotopic injection reviewed in [16]). This relatively easy and reliable in vivo method of pancreatic tumor formation is easier to

genetically manipulate than autochthonous GEMMs, and yields reproducible primary tumor growth and metastasis kinetics. Injection of KCPY cultures gives the added advantage of being able to study metastasis in vivo, as previously discussed. Many studies in the pancreatic cancer field utilize both GEMMs and orthotopic models of cancer to address different aspects of tumor formation and metastatic disseminations, and both tools are great resources for studying the effect of genetic perturbation or pharmacological inhibition.

Mechanisms of Metastasis

EMT-MET Axis and Cellular Plasticity

The KPC and KPCY autochthonous pancreatic cancer models have been used by many in the field to determine the role of epithelial plasticity in PDAC metastasis. The original KCPY study demonstrated that YFP-tagged tumor cells undergo EMT and invade at an early stage to form micrometastases at the secondary site [3]. Subsequent studies have shown that a reversion to epithelial morphology, a phenomenon termed mesenchymal-to-epithelial transition (MET), is required to thrive and form macrometastases at the secondary site [17]. Conventionally, PDAC tumor cells are thought to gain more mesenchymal characteristics by undergoing EMT within the primary tumor, giving them the ability to invade into the tumor parenchyma, as they look for vasculature to begin their metastatic journey. After intravasating into blood vessels, tumor cells maintain their mesenchymal status and travel through the circulatory system, until they reach their eventual metastatic site, at which point they must extravasate into the secondary organ and undergo mesenchymal-to-epithelial transition (MET) to re-establish their epithelial identity. Once returned to an epithelial state, the tumor cells will begin to colonize and proliferate in the metastatic site. The prevailing thought is that the EMT–MET axis is very plastic, with tumor cells existing at various states throughout the spectrum in order to survive and thrive in their new environments (reviewed in [18]). Epithelial status is thought to be a pro-proliferative state, while mesenchymal status is a migratory state. Furthermore, cells undergoing EMT are thought to be more drug-resistant with a certain degree of stemness that allows them to thrive in adverse conditions, such as in circulation and when first arriving in foreign organ.

The EMT process is largely regulated at the transcriptional level through various transcription factors such as zinc finger E-box-binding homeobox 1 (ZEB1), twist-related protein 1 (TWIST), zinc finger protein SNAI1 (SNAIL), zinc finger protein SNAI2 (SLUG), and paired related homeobox protein 1 (PRRX1) (reviewed in [18]), although post-translational mechanisms have been invoked recently [19]. Conventionally, these transcription factors are thought to activate an EMT program as well as a stem cell-like program. However, the two processes are not completely linked, as is the case for PRRX1, which uncouples EMT and stemness [20]. Furthermore, PRRX1 has been shown to regulate other forms of epithelial plasticity within the pancreas, such as acinar-to-ductal metaplasia (ADM) [21], which indicate that these transcription factors have various context-specific roles for regulating plasticity at multiple levels. Perhaps more interesting, *PRRX1* has two main isoforms, *PRRX1A* and *PRRX1B*, which promote MET and EMT, respectively [22]. This ability for these two isoforms to regulate both epithelial and mesenchymal states highlights the complex nature of the EMT–MET axis and showcases the plasticity inherent to the system. This has become especially evident in recent years, as various groups have begun to describe partial EMT intermediate or hybrid states. The epithelial state has been historically defined by E-CADHERIN (here E-CAD) and cytokeratins, and the mesenchymal state primarily through N-CADHERIN (herein N-CAD) and vimentin [23]. Therefore, EMT has classically been defined as loss of E-CAD and gain of N-CAD. However, the intermediate EMT states that have been described (i.e., partial loss of E-CAD or co-expression of both epithelial and mesenchymal markers within the same cell), the so-called hybrid epithelial and mesenchymal

phenotypes, have broadened the definition of an EMT cell. Importantly, these hybrid cells can undergo further EMT to become more mesenchymal, or the reverse MET to re-establish their epithelial identity. Indeed, one recent publication posited that EMT is dispensable for metastasis [24].

Metastatic Organotropism: The Role of Exosomes and EMT Modulators

Metastatic organotropism may have first been described over a century ago by the English surgeon, Stephen Paget, when he elegantly described a non-random pattern of metastasis in over 700 women who died from breast cancer [25]. Paget went on to postulate that tumor cells are a seed that will only propagate when they fall on congenial soil. This provocative idea continued to perplex biologists for much of the last century, as some have argued that tumor cells have intrinsic properties that help it choose the secondary organ, while others have shown that the metastatic site itself provides an environment that is favorable for growth. This debate is particularly well suited for PDAC, as it is one of the most metastatic cancers, with upwards of 80% of pancreatic cancer patients presenting with metastases [26]. In PDAC patients, the two most common sites of metastases are the liver and the lung, and recent evidence has begun to elucidate mechanisms that may determine liver-versus lung-tropic programs. This may be of particular clinical importance for PDAC patients, as those with isolated pulmonary recurrence after pancreaticoduodenectomy have significantly increased overall survival compared with patients who have metastases to other sites (40.3 months versus 20.9 months, respectively) [27]. A separate study corroborated this finding in an independent cohort by demonstrating that PDAC patients with lung-only metastases had a median survival of 31.8 months, while those with liver-only metastases survived 9.1 months [28]. Therefore, we will delve into the underlying processes that have been proposed to control metastatic organotropism in PDAC.

Tumor exosomes, small membrane vesicles (30–100 nm) secreted by tumor cells, are packaged with a plethora of biological molecules including, but not limited to, DNA, RNA, miRNA, and protein (reviewed in [29]). Exosomes produced by the primary tumor are able to enter the circulation and have been shown to set up a pre-metastatic niche in secondary organs prior to tumor cell arrival and seeding. This pre-metastatic niche alters the local microenvironment to make it conducive for growth of the parental exosome-producing cell. Thus, to extend Paget's analogy, exosomes are secreted by the seed and fundamentally change the soil, thus priming it for implantation of the seed. In PDAC, this has been shown elegantly by David Lyden's group, where they established that tumor-derived exosomes are taken up by resident cells at the metastatic site to prepare the pre-metastatic niche [30]. Specifically, they demonstrated that the exosomal integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ were associated with lung metastasis, while exosomal integrin $\alpha_v\beta_5$ with liver metastasis. Thus, their work has established that pre-metastatic niche formation by exosomal education is a potential mechanism of metastatic organotropism in PDAC. Furthermore, this work has led to the premise that identification of sub-populations of tumor-derived exosomes in circulation may help to predict eventual sites of metastases, and that increased monitoring to those sites may be beneficial. A related study by Achim Krüger's group identified Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) as a pre-metastatic niche modifier in the hepatic microenvironment and showed that patients with high plasma TIMP-1 have increased incidence of liver metastases [31]. It is not yet undetermined if TIMP-1 is a global pro-metastatic molecule (i.e., promotes all metastases, and not just liver-tropic metastases), as the authors did not discuss potential roles in other metastatic sites, outside of the liver. However, the proposed mode of action for promoting liver metastasis by TIMP-1 is that it activates hepatic stellate cells to prime the liver for metastases, and we speculate that this is a liver-tropic mechanism of metastasis and would not apply to the resident lung environment. Future studies might identify that other secreted factors are able to act on resident lung cells as potential lung-tropic mechanisms.

Another proposed mechanism of liver versus lung metastatic tropism is that internal mechanisms within the tumor cell give it unique characteristics that will determine the liver- or lung-tropic phenotype. Specifically, we have shown that genetic deletion of either P120CTN (encoded by *Ctndd1*) or E-CAD (encoded by *Cdh1*) in KPCY mice forces tumor cells to undergo EMT and dramatically increases metastatic load specifically to the lung [32]. This is in stark contrast to control KPCY mice, which primarily metastasize to the liver, at a much lower rate. We propose that tumor cells that metastasize to the liver require P120CTN-mediated restabilization of membranous E-CAD, in order to undergo MET and colonize the liver. Cells that lack P120CTN or E-CAD are unable to undergo MET and exclusively metastasize to the lung, which allows for metastatic colonization in cells that maintain a mesenchymal state (i.e., cells that have undergone EMT, but cannot revert to an epithelial state through MET). Taken in the context of the potential hybrid EMT cells discussed earlier, it is likely that, in an evolving tumor, subpopulations of cells exist that are at various stages of the EMT–MET axis, and that these cells may seed the lung if they are more mesenchymal and the liver if they are more epithelial.

We do not propose that these are the only mechanisms of metastatic organotropism, and likely that many systems are likely at play de novo in PDAC patients. It is tantalizing to speculate that the cell-intrinsic mechanisms that drive tropism (i.e., P120CTN or E-CAD protein loss in the primary tumor) could give rise to two different cell populations that interact with their premetastatic niche in unique ways. In this scenario, cells which have undergone EMT, and are therefore possessing mesenchymal characteristics, might have increased expression of lung-tropic exosomes with integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$. On the opposite side of the spectrum, cells that can undergo MET and thus possess epithelial characteristics might express exosomes with $\alpha_v\beta_5$ and metastasize to the liver. This may, in part, explain why some patients present with both liver and lung metastases, as their heterogenous primary tumor may be comprised of cells with both epithelial and mesenchymal characteristics, which simultaneously send both liver-tropic and lung-tropic exosomes to their secondary sites, preparing both for colonization. It is likely that these and many other mechanisms of metastatic organotropism are working simultaneous and that their interplay is what ultimately determines the site of metastasis.

Epigenetic and Post-Transcriptional Regulators of EMT and Metastasis

As previously mentioned, EMT has classically been described as a transcriptionally regulated process through EMT-transcription factors (EMT-TFs herein). However, recent data in PDAC have started to shift this paradigm by establishing that EMT can also be regulated through epigenetic and post-transcriptional mechanisms.

The primary means of regulating PDAC EMT at the epigenetic level have been through histone modifications, DNA methylation, and miRNA-mediated control of canonical EMT-TFs (reviewed in [33, 34]). Histone Deacetylase 1 and 2 (HDAC1 and HDAC2 herein) have been the most well-studied deacetylases that facilitate the epithelial plasticity observed PDAC, mostly in regard to their silencing of E-cadherin (*CDH1*) expression. This silencing is observed in highly metastatic PDAC cells and is mediated through a transcriptional repressor complex between SNAIL and HDAC1/2 that hones to the *CDH1* regulatory elements [35]. A similar study showed that recruitment of the HDAC1/2 to the *CDH1* promoter can also be accomplished by ZEB1/HDAC repressor complexes [36], indicating that this may be a more generalized mechanism of regulating epithelial identity in PDAC. In the absence of SNAIL or ZEB1, *CDH1* remains acetylated and silenced, as HDACs are unable to be recruited to the promoter [35, 36]. Clinically, the class I HDAC inhibitor, mocetinostat, has the ability to reverse EMT by interfering with ZEB1 function [37]. This is not unique to HDAC inhibitors, as various small molecular inhibitors of epigenetic readers, writers, and erasers have shown promise in PDAC GEMMs

previously discussed (reviewed in [38]). High expression of the histone methyltransferase enhancer of zeste homologue 2 (herein EZH2), in particular, has been shown to promote PDAC cell plasticity and to be a poor prognosis indicator in PDAC patients [39]. Genetic deletion or pharmacologic inhibition of EZH2: enhanced the anti-proliferative effect of gemcitabine, reversed EMT, and inhibited cellular migration in PDAC cells [40, 41]. Collectively, these studies have demonstrated that many different histone modifiers have the ability to modulate cellular plasticity in PDAC and targeting these molecules may be an Achilles heel in the EMT cascade. One of the most well-studied routes of miRNA-mediated epigenetic regulation of EMT in PDAC is through the miR-200 family. The p53-miR-200c axis has been studied in PDAC, where loss of p53 downregulates miR-200c, which alleviates normally represses the EMT program by degrading EMT-TF mRNAs [42, 43]. The broader miR-200 family appears to have similar roles in negatively regulating EMT [44–46], and overexpression of miR-200a or miR-200b in PDAC cells downregulated EMT-TFs [47], suggesting a conserved mechanism. All of these processes being described fundamentally alter the expression levels of critical EMT regulators, and either suppress or enhance EMT.

Ben Stanger's group has recently provided some provocative work demonstrating that regulation of E-CAD protein level and localization is important for the degree of plasticity achieved during EMT. To that end, they established that two distinct EMT programs exist in PDAC: complete EMT (C-EMT) and partial EMT (P-EMT) [19, 54]. C-EMT was shown to be primarily driven through transcriptional repression of the epithelial program (i.e., downregulated expression of classical epithelial genes like *Cdh1*), while P-EMT maintained epithelial cell identity transcripts, but functionally altered the protein products of these genes. Specifically, P-EMT is mediated by re-localization of E-CAD from the membrane to the cytoplasm, causing cells to lose their epithelial cell qualities. Ultimately, both sub-types turn on mesenchymal gene programs during their respective EMTs, but repressed their epithelial cell identity through different mechanisms. Interestingly, both types of EMT cells were able to undergo MET, but differed in their invasive and metastatic qualities. P-EMT cells maintained their cell-to-cell contacts and invaded as clusters of cells, while C-EMT cells completely lost the ability to form cell junctions and invaded as single cells. This is important in vivo, as it has been known for nearly 40 years that circulating tumor cells (CTCs) that form clusters are more metastatic [48–52], and suggests that P-EMT cell clusters will have enhanced metastatic potential. Furthermore, this model of partial EMT fits well with the previously GEMM data in that P120CTN loss causes re-localization of E-CAD protein from the membrane to the cytoplasm, akin to the P-EMT [32]. Importantly, others have shown that PDAC patients who have cytoplasmic staining of P120CTN have significantly decreased survival relative to those with membranous P120CTN [53], which may mark a patient population undergoing partial EMT.

Concluding Remarks

We have discussed various means of regulating epithelial cell identity in PDAC and have introduced emerging paradigms that are re-shaping the broader EMT field. The publications reviewed herein have truly re-invigorated the field of EMT research in pancreatic cancer, and new insights will hopefully translate into new approaches for early detection, risk stratification, and therapy.

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Current Perspectives on Nasopharyngeal Carcinoma

Hui Mei Lee, Kazuhida Shaun Okuda, Fermín E. González, and Vyomesh Patel

Abstract

Of the ~129,079 new cases of nasopharyngeal carcinoma (NPC) and 72,987 associated deaths estimated for 2018, the majority will be geographically localized to South East Asia, and likely to show an upward trend annually. It is thought that disparities in dietary habits, lifestyle, and exposures to harmful environmental factors are likely the root cause of NPC incidence rates to differ geographically. Genetic differences due to ethnicity and the Epstein Barr virus (EBV) are likely contributing factors. Pertinently, NPC is associated with poor prognosis which is largely attributed to lack of awareness of the salient symptoms of NPC. These include nose hemorrhage and headaches and coupled with detection and the limited therapeutic options. Treatment options include radiotherapy or chemotherapy or combination of both. Surgical excision is generally the last option considered for advanced and metastatic disease, given the close proximity of nasopharynx to brain stem cell area, major blood vessels, and nerves. To improve outcome of NPC patients, novel cellular and in vivo systems are needed to allow an understanding of the underling molecular events causal for NPC pathogenesis and for identifying novel therapeutic targets and effective therapies. While challenges and gaps in current NPC research are noted, some advances in targeted therapies and immunotherapies targeting EBV NPCs are discussed in this chapter, which may offer improvements in outcome of NPC patients.

Keywords

Asian cancer · Nasopharynx · Epithelial cells · Therapies · Cell lines · Animal models

Introduction

Historic Perspective

As reviewed by Muir CS, early cases of nasopharyngeal carcinomas (NPC), circa nineteenth century, were very often poorly described, misdiagnosed, or misclassified. This was broadly due to the poor understanding of the disease and the associated symptoms at that time. For example, while symptoms of enlarged neck glands,

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blocked nose including hemorrhage from the nose, and difficulty in hearing were recorded, diagnosis were often made without histopathological evaluation leading to misdiagnosis of symptoms that included among many, small cell medullary cancer. Classifications were equally confounding and classified for example, as medullary cancer invading lymph tissue, epithelioma to lymphoepithelioma due to lymphocytic infiltrate in the lesions. Also, mentioned were some of the early treatments used for these misdiagnosed cases. Treatment ranged from radical surgery, use of nitric acid, opium, and ligation of the carotids. Etiology of these early cases of NPC was based on opinions and speculations and broadly focused on smoke exposures from sources such as tobacco and opium smoking and candles. In China, NPC was described as cancer of the neck glands, but it was not until early twentieth century that the first diagnosis of NPC was made. The diagnosis made was that the cancer's origin was the epithelium of the nasopharynx and noting that this cancer type was unusually predominant in large parts of China and migrant Chinese communities residing in nearby locations (Singapore, Indonesia) and more distant locations (USA, Europe) which suggested an underlying genetic basis for this disease [1]. However, past data have also reported elevated incidence rates of NPCs among the Malays and Dayaks indigenous population of Borneo which were comparable to the Sarawak Chinese, in non-Chinese mongoloid populations of Vietnam and Thailand, and in certain countries of the African subcontinent, which suggested a viral or environmental etiological factor for this disease [2, 3]. In support, recent data noted a decline in NPC rates in Chinese migrants living in low-risk countries (Peru, Canada, USA) further suggesting a change in life style habit from exposures to more traditional diet of, for example, salted dried fish and further mentioned below in this chapter [4]. Other compounding factors that hindered advancing our knowledge on NPC included past estimates of new cancer cases. For 1980, these were limited to ~24 geographic locations and to 16 common cancer sites, with several cancers classed as groups. For example, oral and pharyngeal cancers were classed as a group, as classifications to further subdivide them was not harmonized. Consequently, in high-risk populations of Hong Kong and Singapore for developing NPC for example, NPC cases were omitted from the 1980 estimates in order to obtain figures for the broad oral-pharyngeal group. Pertinently, estimated total number of cancer cases which did not include NPC were estimated to be at ~6.35 million [5]. Furthermore, lack of Ear Nose and Throat (ENT) departments in these geographic regions was likely the reason for NPC cases being under reported or not fully evaluated to included cervical lymph nodes for making a definitive diagnosis [2].

Current Perspectives

More recent GLOBOCAN cancer estimates now include NPC as a cancer site, distinct from other head and neck cancers (lip, oral, larynx, oropharynx, hypopharynx) among the 36 cancer sites evaluated. Using data available from the most recent report, an estimated 18.1 million new cases and 9.6 million cancer deaths will be recorded for 2018 worldwide. Importantly, almost half of these cases and cancer deaths for 2018 will be in countries in Asia, where ~60% of the world population reside and exposed to dietary and environmental risk factors. From these estimates, ~129,079 new cases and 72,987 deaths will be attributed to NPC and as alluded to, most of these cases will be confined to countries in Asia [6]. Risk factors for NPC still remain poorly understood, however, those that are now thought to be key, are further discussed in detail in this chapter. Also, early symptoms of NPC like most other cancers are asymptomatic and include headache, neuropathic facial pain, cervical mass, epistaxis, or nasal obstruction, resulting in misdiagnosis, delay in treatment, and poor outcome. The current standard of care for suspicious NPC in at-risk populations, calls for a full endoscopic evaluation of the upper aerodigestive tract for a diagnosis followed by treatment. Recent data suggest that misdiagnosis of NPC patients with symptoms of headaches for example, was

~43.4%, and even with endoscopy median time from presentation of symptoms to treatment was ~6 months and most notably ~30% of the NPCs were missed with nasal endoscopy. Collectively, there is an unmet need for improved awareness of risk factors, presenting symptoms, and limitations associated with diagnostic evaluations, to prevent misdiagnosis, improve early detection for receiving treatment that will likely confer a favorable outcome [7, 8]. It is worth mentioning that in some regions of SE Asia, time between diagnosis and start of radiotherapy as first-line treatment was ~120 days resulting in a negative impact on the overall survival of NPC patients, which was partly attributed to unaffordability of the treatment, but the major contributor nonetheless, was the limited number of radiotherapy facilities available [9].

NPC is a malignancy that arises in the outer most epithelium lining of the nasopharynx cavity located above the oropharynx and hypopharynx, in close proximity to the base of the skull. The cancer shows a variable degree of squamous differentiation where the vast majority of tumors that present in Asian countries are undifferentiated without evidence of keratinization and can be classified as non-keratinizing undifferentiated with mild lymphatic infiltration or non-keratinizing differentiated NPCs (Fig. 1a, b). As with most cancers, the stage of the disease is a key prognostic factor that impacts outcome and survival. Recent data shows that early stage NPC treated with radiotherapy alone responds most favorable but lesions with disease progression; locoregional and distant recurrences impact the 5-year overall survival rates which drop from ~90% to 75% for stage I and stage II diseases. Also, treatment prescribed for lesions with progression is concurrent chemo-radiotherapy which can result in toxic side effects. Prognosis is further compromised if NPC is diagnosed with cervical or retropharyngeal nodal metastases as these patients may have increased risk for distant

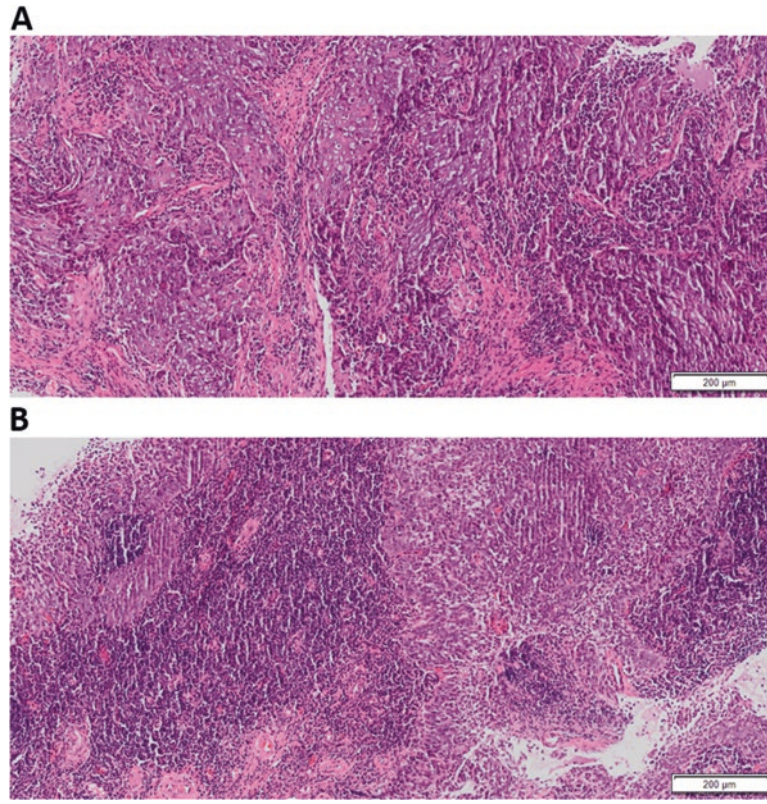

Fig. 1 Hematoxylin and Eosin (H&E) staining of common NPC cases that present in Asia and are sub-grouped into (**a**) non-keratinizing undifferentiated with mild lymphatic infiltration and (**b**) non-keratinizing differentiated lesions

micro-metastases that would also require concurrent chemo-radiotherapy. Notably, late stage IV disease can account for ~10% of all NPC cases in at-risk regions with data showing that this patient cohort can have a 1-year survival rate of ~48% with standard-of-care chemo-radiotherapy [10].

Broadly, NPC remains a major public health concern, given that number of new cases of NPCs and deaths due to the disease are likely to increase within Asia, coinciding with an increase in world population. While advances have been made with intensity-modulated radiation therapy (IMRT), options for chemotherapy remain unchanged. As already mentioned, a better awareness and understanding early symptoms of NPC will enhance early detection and almost immediate treatment with IMRT and improve outcome of NPC patients. Also, reduced exposures to likely risk factors such as incomplete combustion of fossil fuels and wood fires used in cooking and food processing (salted dried fish) leading to smoke particulates being trapped in the nasopharynx can likely impact incidence rates of NPCs. This chapter will cover current perspectives on incidence rates of NPC and likely risk factors, for example, Epstein Barr virus (EBV), that are known to be associated with the disease and the impact of migration of at-risk population. Importantly, headwind in knowledge gaps and key challenges in NPC *bench-to-bedside* translational research in the context of model systems will be discussed. Finally, immunotherapy as approach for NPC therapy and novel zebrafish models that may have value for identifying novel therapies for NPC, with the view of improved understanding of the molecular basis of human NPC development and treatment options for improved outcome, will be mentioned.

Epidemiology

Incidence Rate

Based on variation in genetics of different subpopulation and cultural practices that influence eating habits, certain cancers have a higher prevalence in some ethnic groups in different parts of the world. One of the cancers with unique ethnic and geographical distribution is NPC. As already mentioned, this cancer most commonly starts in the epithelium lining of the nasopharynx, an area that is ~4 cm in length and 3 cm width located at the back of the nasal cavity and toward the back and base of the skull. NPC is a subset of head and neck cancers and can be categorized into keratinizing NPC or non-keratinizing NPC [11]. This cancer is highly prevalent in China in Provinces within the East to South Eastern region of the country. For example, Guangdong, Guangxi, Zhongshan, and Hong Kong have an age-standardized incidence rate per 100,000 population (ASR) at more than 10 [12]. Furthermore, Zhongshan and Zhuhai have some of the highest ASR at 25 and 24, respectively, twice the ASR in comparison to nearby states. Collectively, human population of Chinese descendants residing in South East Asia have intermediate risk with the highest ASR in Brunei at 13.6, followed by 10.6 in Malaysia and 9.5 in Singapore. In other countries in South East Asia such as Philippines, Vietnam, and Thailand, the ASR is ~3 to 5. Interestingly, population residing in the African subcontinent have a moderate risk of developing NPC with ASRs of ~5.9 for Sétif Province in Algeria and 4.4 for Nairobi in Kenya. In other regions of the globe, for example, North America, Europe, Latin America, Caribbean, and Oceania by contrast, NPC is considered as a rare disease with an ASR of less than 2. However, certain native indigenous groups in low-risk regions have elevated risk at an order of magnitude equivalent of endemic regions, for example, ASR of Nunavut region in Canada is ~15.2, while for Alaska's indigenous natives, USA, and Pacific Islanders in New Zealand, ASRs are 6.2 and 5.5, respectively [12]. These data strongly suggest a role of genetic susceptibility for developing NPC. Lastly, gender also has an impact on the ASR of NPC where males are threefold more likely to develop NPC compared to female [13]. As already mentioned, close to 60% of new cases of NPCs

and associated deaths for 2018 will occur within Asia [6]. Global rates of NPC including those occurring in Asian countries and within ethnic sub-populations are depicted in Fig. 2a.

Impact of Human Migration on NPC Incidents

In an era of globalization where migration occurs to seek improved life quality, career, and education, dynamics of cancer incidences can change

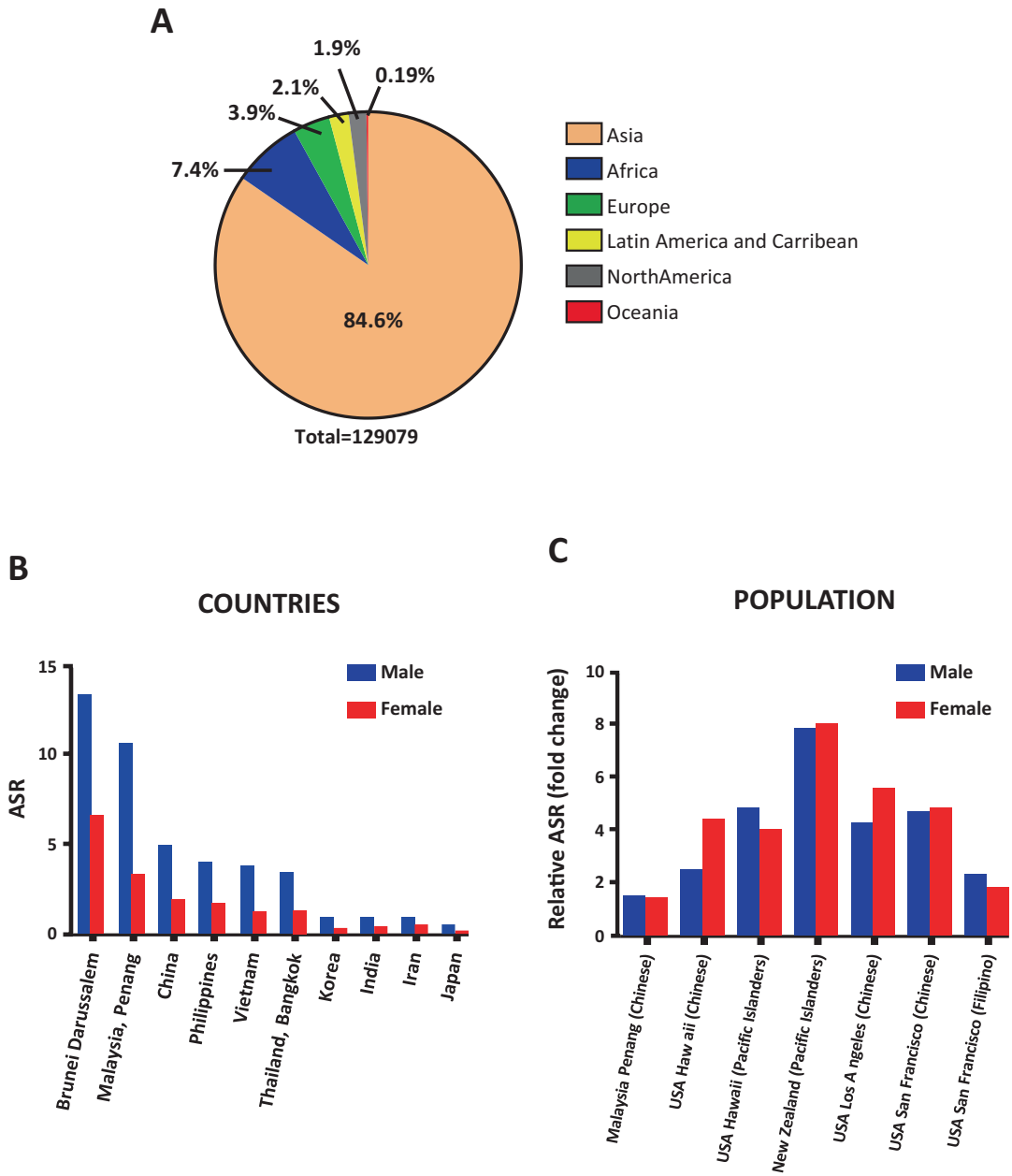

Fig. 2 (**a**) Incidence rates of NPC estimated for 2018. (**b**) Age-standardized rates (ASR) of NPC in Asia and (**c**) relative fold change ASR within migrated ethnic sub-populations compared to the overall population of the area. Adopted from GLOBOCAN 2018 and Bray et al. [6]

as well. For domestic migration, people generally move from poorer rural areas to the cities. For example, in China NPC incidence was noted to be ~2.23 in urbanized areas, an ASR that was marginally higher when compared to rural areas where the ASR was ~2.07 in 2013. This was attributed to lifestyle that leads to increased exposures to risk factors (salted dried fish, environmental toxicant) as well as better data quality in cancer registries at cancer centers in major hospitals of large cities [14]. For international migration, migrants from endemic regions continue to have higher risks of developing NPC after migration to non-endemic regions of the world. For instance, the incidence rate of NPC in Chinese in USA is ~tenfold higher than the general US population. Similarly, ASR of immigrants from South East Asia residing in Sweden is ~30-fold higher [12, 15]. While underlying genetic factors contributing to the disease still remain to be elucidated, environmental factors also have a detrimental impact as the second and third generation of Chinese in California, USA, show reduced risk of NPC as compared to their parents [16]. Nonetheless, migration also affects the incidence of the histopathological type of NPC in low-risk countries. For example, incidence of keratinizing NPC which is more prevalent in low-risk regions and among the Caucasian population showed a gradual reduction from 47% to 35% from 1989 to 2009 in the Netherlands. On the other hand, incidence of non-keratinizing NPC has increased steadily from 45% to 59% over the same year span [17]. Of interest, studies have shown that Caucasian descendants had higher risks of developing NPC after relocating to endemic areas [18, 19]. Figure 2b, c depicts the latest (2018) incidence rates of NPC within Asian countries and migrated ethnic sub-populations.

Risk Factors

Epstein Barr Virus (EBV)

NPC is intimately associated with exposure to the Epstein Barr virus (EBV). EBV is a ubiquitous virus infecting ~90% of the world population, yet only a fraction of population develops cancer-related to EBV, considering that B-cells and epithelial cells are prone to EBV infection. In B-cell-related cancer, EBV infection can be detected in up to 40% of Hodgkin lymphoma and ~95% in Burkitt lymphoma especially in regions where malaria is endemic [20, 21]. As for solid epithelial cancers, almost all patients diagnosed with undifferentiated NPC and ~10% of gastric cancer cases are EBV-positive [22]. As well as undergoing H&E histopathological evaluation, NPC biopsies are also assessed for the presence of EBV. This is achieved by probing for the EBV-encoded RNA (EBER) by in situ hybridization (ISH), and the non-isotopic probe gives a purple signal that is localized to the nuclei of affected cells and an indication of viral copy number. A representative EBER ISH staining of a confirmed NPC biopsy is shown in Fig. 3. Due to this causal link for a subset of cancers, the EBV genome has been actively investigated since the 1980s to better understand the molecular characteristics and the open reading frames of the virus which encode genes that could be responsible for NPC development. Of interest though, recent data now show that the EBV genome from different geographic locations share high similarity within the open reading frames with the exception for the differences noted in the open reading frame that encodes EBNA2 and EBNA-3, and consequently categorized as either EBV-1 or EBV-2. Notably, EBV-1 is common in most parts of the world, whereas EBV-2 is now known to be prevalent in African subcontinent. Although the genetic differences in these two EBV genomes are small, recent data demonstrated that a single amino acid change from serine to aspartate in EBNA2 of EBV-2 was sufficient to enhance the growth of B-lymphoblastoid cell line similar to cells infected by EBV-1 [23–26].

To further understand how minor genomic diversity in EBV contributes to different types of cancer across geographical regions, a recent study sequenced the EBV genomes isolated from patients diagnosed with different EBV-positive cancers. Principal-component analysis (PCA) of single nucleotide polymorphisms (SNP) identified in different EBV genomes demonstrated that EBV

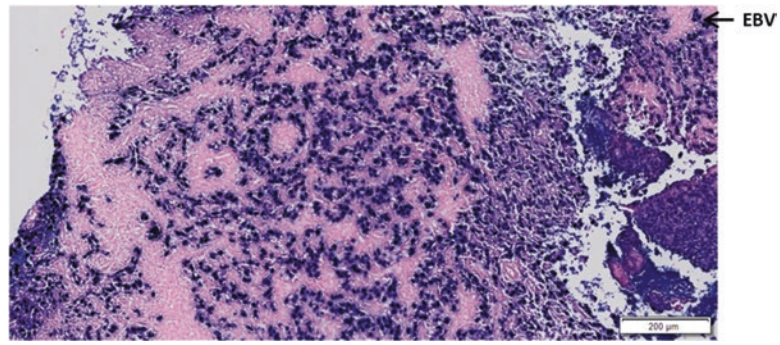

Fig. 3 Detection of EBV-encoded RNA (EBER) in a confirmed NPC biopsy using non-isotopic in situ hybridization. Presence of EBV is confirmed by the purple staining and indicated by arrow

of Asian origin was clustered separately from other geographic regions. Furthermore, the authors also proposed that these minimal differences in EBV genome of Asian origin could be a contributing factor in NPC development compared to EBV of other origins [27]. In 2017, Tsai and colleagues further explored the infection and tumor induction efficiencies of the different strains of EBV. They demonstrated that YCCEL1, a cell line established from a Korean gastric cancer patient positive for EBVaGC (or otherwise known as M81), an EBV subtype with enhanced pathogenic potential isolated from a Chinese patient diagnosed with NPC, could readily and efficiently infect respiratory epithelial and gastric cancer cells when compared to EBV producing Akata cells isolated from Burkitt lymphoma and the non-human primate EBV shredding B95-8 cells [28, 29]. It was suggested that the high expression of the viral glycoprotein, gp110, could enpower EBV with properties of tropism to enhance infection efficiency of the virus in epithelial cells [28]. Besides differential expression of glycoproteins, the infection phase of EBV should also be noted. EBV infection can be latent or lytic. During latent infection, the virus only replicates when the cell is dividing, whereas during lytic infection, linearized EBV genome with various number of tandem 500 base pair (bp) repeats at each terminus is being packaged as virus particles for transmission from saliva to epithelial cells. Once inside the epithelial cells, EBV genome does not integrate into the host genome, but the terminals of the virus fuse together to form episomes and consequently the infection remains as latent infection. Detection of fused terminal fragments with similar sizes from NPC biopsies suggests that NPC may start from a clonal expansion of a single EBV-positive cell [30]. Currently, the molecular basis associated with EBV pathogenesis in the nasopharyngeal epithelium remains largely unknown.

Single Nucleotide Polymorphisms (SNPs)

To date, more than 100 genome-wide association studies (GWAS) have been carried out to identify single nucleotide polymorphisms' (SNPs) genetic variants that may lead to the development of NPC. One of the most consistent finding is that certain *human leucocyte antigen* (HLA) class I in the major histocompatibility complex (MHC) is associated with NPC. Several notable studies have shown that the presence of HLA-A-0207, A-3303, and B-5801 was associated with a higher risk of developing NPC while HLA-A-1101 was associated with a lower risk [31, 32]. Allele frequency of HLA-A∗02:07, A∗33:03, and B∗58:01 is also higher in endemic regions of SE Asia, with a range from 1% to 15% when compared to USA, European Caucasians, where the frequency is less than 0.03% [33]. This could partly explain why this disease has a unique geographical distribution. Moreover, HLA-A was recently reported to be one of the significantly mutated genes in NPC patients, highlighting a potential causal role of this gene in the development of NPC [34]. To further verify and validate these observations, larger GWAS studies involving a larger patient cohort are warranted.

The genome of each cell in the human body consists of deoxyribonucleic acid (DNA) that codes the genetic instructions that are needed at the cellular level to make proteins crucial for maintaining the cells' normal biochemical activities [35]. However, as a caveat, the genome also represents a critical target for exposer to DNA damaging assaults irrespective of whether these have an environmental origin or abnormal cellular metabolites, for example, reactive oxygen species. To counter this, the human genome has evolved to include ~130 genes from the estimated ~17,294, the protein products of which play a key role in the DNA repair process to maintain DNA integrity in the case of, for example, DNA double strand breaks through homologous recombination and non-homologous end joining [36, 37]. It follows that ineffective DNA repair mechanism can likely increase the risk of developing cancer in venerable populations exposed to EBV and increased susceptibility of developing NPC. In a notable study, the authors genotyped DNA from ~2349 NPC patients from Hong Kong, for ~377 SNPs spanning 161 genes and loci and of these 266 SNPs matched to 81 DNA repair genes [38]. The rs401681 SNP located in the intron of *CLPTM1L* at *TERT-CLPTM1L locus* of 5p15.33 and double strand break repair pathways were the most significant indicators of elevated risk of NPC. In another case study from Hong Kong, the authors performed GWAS of EBV DNA isolated from saliva of healthy carriers and NPC tumor biopsies and identified a panel of NPC-associated SNPs and indels in the EBER locus including a four-base-deletion polymorphism downstream of EBER2 (EBER-del), suggesting that these EBV variants could explain for the high incidence of NPC in high-risk areas of SE Asia [39]. Of note, in other GWAS studies SNPs identified in *CLPM1L-TERT* locus, *MECOM, TNFRSF19*, and *CDKN2A/B*, and in genes in the TGF-β and JNK signaling pathways were associated with increased risk of NPC and other cancers [40–44]. However as proposed by Boyle et al., while essential genes or essential pathway contribute to the phenotype of the disease, genes affecting the risk of the disease could also be driven by the expression of a combination of variants outside the essential pathway with small effects and ultimately to disease development [45].

Intake of Certain Foods

Reports now suggest that the consumption of a traditional diet consisting of salted fish and rice, popular among the poorer rural Chinese communities from an early age, could be a major contributing factor for the high incidence rates of NPC. Epidemiological data from Hong Kong and Malaysian Chinese in Malaysia now suggests that occupational dust and inhalant exposure and salted dried fish consumption during childhood and into adult were major risk factors for NPC among Chinese immigrants [46]. This was further supported from a study that evaluated the medical history of Chinese NPC patients for dietary habits, occupational exposures, use of tobacco, and alcohol. When the data was compared to control subjects, exposure to salted fish was associated with an increased risk of NPC and independent factors likely contributing were identified as an early age of exposure, increased duration and frequency of consumption, and cooking the salted fish by steaming as opposed to other methods such as frying, grilling, or boiling [47]. The process of preparing salted dried fish in South East China involves salting with crude salt and immersing in brine followed by sun-drying and storage for 4–5 months before consumption. High temperature and humidity can increase the risk of bacterial growth, for example, *Staphylococci*. During the preparation of salted fish, key carcinogenic N-nitroso compounds including N-Nitrosodimethylamine are formed from the naturally occurring secondary amines in the fish, nitrate as a contaminant in the crude salt, and by the presence of nitrate-reducing staphylococci or other bacteria. Studies have demonstrated that N-nitroso compounds undergo metabolic conversion into reactive intermediates that induce DNA damage to initiate cellular transformation that can culminate in cancer. Reports now suggest that exposures to N-nitroso compounds from different sources, for example, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

and N-nitrosonornicotine formed from fermented tobacco and those from certain occupations as alluded to above, have elevated the risk of developing cancers of the upper respiratory tract. N-nitrosodimethylamine can induce tumors in the nasal cavity of experimental animals, and other chemical carcinogens could act synergistically, for example, with EBV to increase the risk of developing NPC [48–50]. It is important to note that most recent analysis of contamination of Chinese salted fish with volatile N-nitrosamines remains high in over ~68% of the samples tested, exceeding the permissible Chinese and USDA limits of 4 μg/kg and 10 μg/kg, respectively. The data suggest that a continuum of risk of developing NPC remains and a need to have a standardized process of preparing salted dried fish that may minimize levels of toxic N-nitrosamines and reduce NPC incident rates [51].

Model System for Investigating Nasopharyngeal Carcinoma

While the survival rates of NPC have improved if detected and treated at early stage of disease development, presence of local residual disease and recurrences which unfortunately is a major challenge in regions that have high incidences of NPC, and limited radio-therapeutic and surgical facilities hamper the overall survival [52]. Further compounding this is that our fundamental understanding of the molecular basis of NPC development still remains poorly understood. This is broadly attributed to the lack of suitable model systems to allow in-depth preclinical investigation to improve our understanding of this cancer. The small subset of the most commonly available NPC cell lines were established several decades ago, for example, CNE-2 was first reported in 1983, as an epithelial cell line established from a poorly differentiated NPC [53]. Other lines available included C666-1 and HK-1 for example, and these were readily shared within the NPC and as well as the broader scientific community, without concerns of authentication. Prior to major concerns with cell line cross-contamination and mislabeling being raised, majority of the NPC lines (CNE-1, CNE-2, HONE1, AdAH, NPC-KT) have been subsequently found to be HeLa contaminated and thus compromised [54–56]. Clean stocks of these cross-contaminated lines have not been identified, which further compounds limitations on advancing preclinical investigation on NPC pathogenesis. A concern remains that these aforementioned contaminated cells lines may be used as cell line models for testing efficacies of promising drugs and for investigating the molecular changes involved in NPC pathogenesis. Thus, it is worth to mention that it is a mandatory requirement of scientific journals for cell line authentication prior to experiments [57]. Irrespective of compromised NPC lines, their scarcity and long-term passage and use in in vitro studies can result in genetic drift, further adds limitations in our quest to improving our understanding of NPC pathogenesis [58].

Furthermore, NPC is also closely associated with EBV. A NPC model system natively infected with EBV is required to study host–viral infection and the underlying molecular changes associated with NPC development [59]. From those available non-compromised NPC lines, C666-1 as reported in 1999 was established from an undifferentiated NPC and was found to consistently retain EBV in long-term cultures and tumorigenic [60]. With still the limited number of NPC cell line model systems available and the associated limitations, a research group in Singapore has been actively involved in establishing NPC cell lines and reported using conditionally reprogrammed conditions using the Rho-associated kinase (ROCK) inhibitor (Y-27632) and irradiated mouse-3T3 fibroblast feeder cells [61]. However, authors noted that these culture conditions preferentially select non-malignant cells to proliferate more efficiently than NPC malignant cells. Given that most of the available NPC cell lines loose the EBV genome after long-term passage, our poor understanding of the pathogenesis of EBV on NPC developments remains a major limitation [62]. Without well-defined culture systems, it remains challenging to advance our knowledge on the most fundamental molecular basis of NPC [62]. More recently, additional two EBV-positive cell lines, C17 and NPC43, have recently been

reported [63]. A key caveat was that Y-27632 was used in establishing C17 and NPC43 line. Key details worth mentioning is that the authors in one of these studies made ~13 attempts to establish NPC cell lines with RPMI medium that was supplemented with 10% FBS and although four outgrowths were observed from the tissue explants, none could be expanded continuously. However, with the addition of Y-27632 in the media, while the rate of explant growth showed almost no difference compared with culture condition without Y-27632, three primary cultures were successfully propagated, and one of the cultures, NPC43, was subsequently found to retain EBV at late passage albeit with a lower EBV copy number. Furthermore, the authors of the study demonstrated that the success of establishing EBV+ cell models was likely depended on the suppression of lytic cycle of EBV [62]. They demonstrated that EBV lytic reactivation was suppressed by Y-27632 at early passage. Upon withdrawal of Y-27632, EBV in a subpopulation of NPC primary cultures was reactivated. The association of ROCK-associated signaling pathways and EBV has been reported in human lymphoma. Burkitt lymphoma and diffuse B-cell lymphoma associated with EBV have been reported to have inactivating mutations in the ROCK signaling pathway [64]. However, it is unclear how the ROCK signaling pathway suppresses EBV lytic reactivation.

While cells grown in monolayer have proven to be extremely useful as cancer model and cost effective, the growth conditions do not always mimic the in vivo microenvironment of human cancers. This limitation effectively renders it difficult to understand certain aspects of the disease such as the composition of tumor microenvironment, effect of tumor microenvironment on cancer cell proliferation, drug metabolism, and cell migration [65]. Furthermore, these limitations may result in many experimental cancer therapies to give the desired outcome in cell line models but failing in in vivo evaluations [66]. To circumvent these limitations, 3D-culture systems have been established to preserve the cell morphology, heterogeneity of cancer, and the associated stroma [67]. In this context, several studies have recently reported the generation of spheroids from NPC cells, for example, using HK1 cells [68]; whereas spheriods cultures of C666.1 using the hanging drop method and the ultra-low attachment method, have all been successfully established and utilized. Also, 2D- 3D-cultures have been reported for NPC and including liquid overlay method, where spheroid cultures of C666.1 using the hanging drop method and the ultra-low attachment method, all have been successfully established and utilized [69–72]. Spheroid cultures have been utilized to better understand NPC cancer stem cells associated with resistant to radio-chemotherapy [73]. It is worth mentioning that these aforementioned model systems do not fully recapitulate the tumor microenvironment of NPC patients. Hence, it is pivotal for future studies to generate spheroid cultures directly from patient's tumor biopsies sample to further understand the causal mechanisms of EBV and NPC pathogenesis [74]. Such approach has been adopted by Scanu et al., who demonstrated that *Salmonella enterica* triggered and maintained malignant transformation using AKT pathway, accompanied by *TP53* mutation and *c-Myc* amplification by co-culturing murine-derived genetically predisposed gallbladder organoids and *Salmonella enterica* [75]. Collectively, further understanding of NPC could likely improve with new spheroid culture methods.

NPC cultures generated from NPC patient-derived xenograft models (PDX) have been reported which include NPC-HK2117, NPC-HK1915, and NPC-HK1530; C15 was derived from a biopsy of a primary poorly differentiated NPC, while C17 and C18 were both derived from metastatic NPC lesions. Isolated cells from these PDX were able to be maintained by the subcutaneous passage into nude mice and the propagated cells retain EBV with C15 retaining the expression of key EBV latent genes encoding EBER, EBNA1, and LMP1 proteins. Thus, these models can be utilized to further enhance our understanding of the role of EBV in NPC pathogenesis and for and preclinical drug evaluation for identifying effect therapies [76, 77]. For example, to identify NPC-specific membrane protein binding partners of LMP1

membrane rafts from C15 cells were used to isolate protein complexes, and using immunoprecipitation and mass spectrometry, galectin 9, a protein elevated in NPC, was identified to be interacting with LMP1, suggesting a casual factor in NPC pathogenesis [78]. C15 was also utilized alongside C666-1, to screen for therapeutic agents for identifying those that have the most promising effect of the cancer cells. From this screen, both cell models were found to be responsive to clinically relevant doses of doxorubicin and taxol, while *cis*-platinum was least responsive [79]. As already mentioned, the establishment of an EBV+ NPC cell line, NPC43, was recently reported [62]. However, it is worth mentioning that this study also reported five additional NPC PDX models by transplanting NPC tissues into NOD/SCID, and newly available resources were used to mine for the underlying genetic landscape the *state-of-the-art* platforms for performing whole exon and whole genome DNA sequencing and RNA sequencing. Data revealed mutations in *NRAS*, *TP53*, *EP300*, and *SMG1* genes. For the oncogene *NRAS*, this was identified as an activating mutation [62]. Collectively, PDX models established several decades ago, and the more recent models can be excellent preclinical model systems to use to screen libraries of drugs and small molecule inhibitors coupled to *high-through-put* platforms to identify the most promising for further validation using in vivo models including zebrafish as described below.

Although in vitro models are invaluable for improving our understanding of NPC, any promising finding requires additional validation in in vivo systems. In this context, in vivo model systems can be divided into cell line xenograft or PDX. Example include HK1 and C666.1 which are tumorigenic when ~10^6 cells are injected subcutaneously into nude mice. Similarly with the newly established NPC43 cell line when co-injected with Matrigel into NOD/SCID mice form tumors [62]. However, a major disadvantage is that these models preclude the investigation of the process of metastasis in NPC pathogenesis. A study of note recapitulated the invasiveness of NPC cancer cells by injecting C666-1 cells orthotopically into the nasopharynx of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice. The resulting tumor demonstrated metastasis to the bone, lung, and the abdomen, broadly mirroring pathogenic features associated with advanced disease in NPC patients [80]. As with PDX models, xenograft models of NPC are reliant on immune-deficient or immune-comprised mice and confer some limitations for modeling human NPC. Notwithstanding, some perspectives on the use of zebrafish for modeling human metastatic NPC are given below.

Potential Use of Zebrafish for Modeling Human NPC

Data now suggests that cancer cells disseminate from the primary site through lymphatic vessels to distant sites. In this context, presence of rich lymphatic network highly correlates with metastatic cancers, for example, melanoma [81]. With no exception, NPC also has a similar propensity for metastasizing to locoregional lymph nodes largely due to the presence of a disproportionately high number of lymph nodes in the neck region and cancer cell-invaded lymph nodes is now widely accepted as the most important factor in NPC prognosis [82, 83]. Thus, it is worth mentioning that to improve our understanding of the invasive and metastatic potential of NPC and to identify promising therapies, robust in vivo models are needed. While orthotopic models of NPC have been developed in mice as mentioned in the section above, they generally fail to show consistent lymph node involvement prior to advancing to a metastatic disease [80]. Zebrafish as a model system offers an attractive in vivo platform for conducting in-depth investigation of human cancers including NPC, primarily due to its low cost of maintenance, rapid developmental speed, and high fecundity rate time. Pertinently, 70% of human protein-coding genes and more than 80% of human disease-related genes have a zebrafish orthologue [84]. This indicates that most molecular mechanisms involved in normal biological processes or in pathologic diseases are conserved between zebrafish and humans and afford an

excellent opportunity to model human cancers. Broadly, there are two ways of modeling human cancer in zebrafish: generating a genetically defined zebrafish strain and xenotransplantation of human cancer cells into zebrafish.

In genetically defined models for example, activating mutations and oncogenes identified from whole genome sequencing of human cancers can be integrated into the zebrafish genome to investigate any pathological effects. This approach has resulted in the development of some notable zebrafish models which has improved our knowledge of human cancers, for example, melanoma. Zebrafish genetic model of melanoma overexpressing the human $BRAF^{V600E}$ under the control of *mitfa* promoter in a $p53^{-/-}$ was found to develop melanocyte lesions that rapidly developed into invasive melanomas recapitulating human melanomas [85, 86]. While this can provide additional information on the molecular process of melanoma development and progression, the models afford an excellent platform for drug screen to identify novel and effective therapies for this lethal form of skin cancer [86]. As large-scale whole genome sequencing of head and neck squamous cancers including NPC has been conducted or under consideration, zebrafish may offer a valuable platform for testing the genetic insights gained from these studies for potentially pathogenic outcome [87–90].

On the other hand, cancer cells isolated from human cancers such as melanoma, colorectal, and breast cancers have been used to successfully xenotransplant into zebrafish embryos [91–94]. Once transplanted into embryos, the cancer cells were then observed to proliferate, form tumor mass, disseminate, and stimulate angiogenesis. Importantly, the optical transparency of zebrafish embryos, coupled with the availability of tissue-specific fluorescent reporters to generate various flavors of zebrafish transgenic lines that fluorescently label cells that are closely associated with human cancer progression, for example, immune or vascular endothelial cells, enables investigators to delineate the interaction between human tumor cells and the zebrafish microenvironment in an in vivo and real-time compartment [95–97]. In this context, a recent study by Follain et al. injected murine mammary D2A1 tumor cell into the zebrafish blood circulation to show that blood flow forces control tumor cell adhesion and extravasation. Furthermore, the authors live-imaged endothelium remodeling at sites where circulating tumor cells adhere, which stimulates extravasation, and have shown that a similar mechanism occurs in murine brain metastasis as well [98]. With the recent development of the lattice light sheet microscopy with two-channel adaptive objectives, biological processes such as cancer cell migration, endothelial adherence, and extravasation can now be imaged in subcellular resolution in zebrafish, further enhancing the utility of the zebrafish cancer xenotransplantation model [99]. Methods to perform patient-derived xenotransplantation in zebrafish had also been established, and recent studies had shown that drug treatment outcomes in these PDX models of zebrafish could accurately predict clinical outcomes in human cancer patients [92, 100–102]. Although a model of inflammatory lymphangiogenesis had been established in zebrafish indicating that zebrafish lymphatics respond to pathological cues, whether human cancer cells interact with zebrafish lymphatic vessels to mirror the early stages of human cancer progress has not been reported [103]. Zebrafish facial lymphatic vessels have been well characterized [97] and therefore xenotransplantation of single cell extracts derived from NPC biopsies, NPC PDX primary cultures of NPC cells from biopsies or established NPC cancer cell lines to the facial region of transgenic zebrafish may reveal novel insights into how cancer cells modulate the local lymphatic environment and disseminate (Fig. 4a–c). The behavior of transplanted

Fig. 4 (continued) facial region of the zebrafish (white dotted brackets) to investigate the interaction between NPC cells and facial red lymphatic/green blood vessels. (**b**, **c**) Lateral and ventral confocal images of the facial region of 5 dpf *Tg(fli1a:nEGFP)*^{y7}*;Tg(lyve1b:DsRed)*^{nz101} transgenic. (**b′**, **c′**) The *Tg(lyve1b:DsRed2)*^{nz101} expression visualizing the lymphatic vessel seen in red of panels (**b**) and (**c**). Confocal z-stack images 5 μm apart were taken using the Zeiss LSM 710 inverted confocal microscope and were processed using ImageJ. *LFL* lateral facial lymphatic, *MFL* medial facial lymphatic, *OLV* otolithic lymphatic vessel, *LAA* lymphatic branchial arches. Scale bars: 100 μm

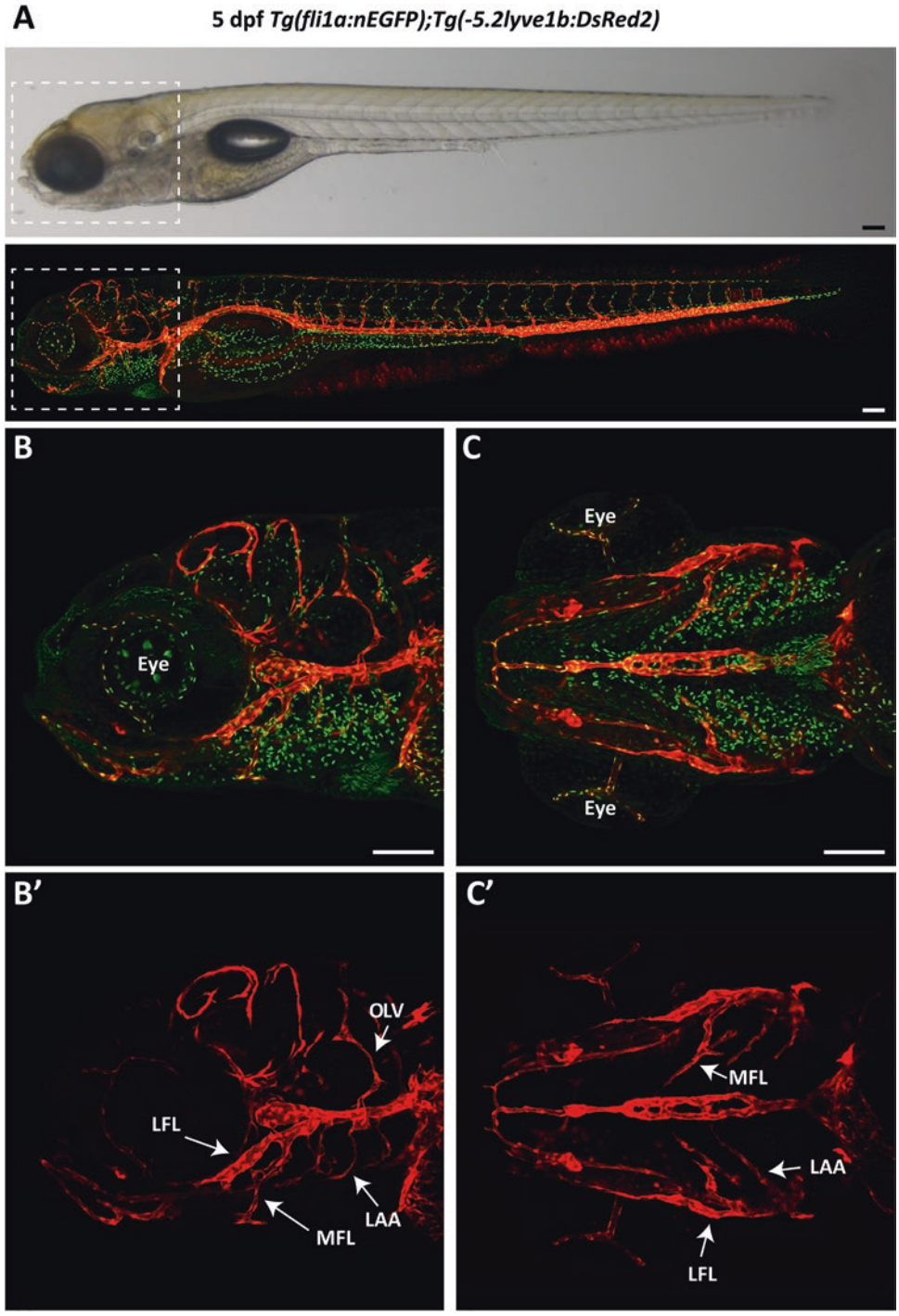

Fig. 4 Zebrafish vascular transgenic strains offer a platform to investigate the interaction between NPC cells and lymphatic/blood vessels for investigating NPC metastasis and identifying promising therapies. (**a**) Upper panel is a lateral bright field image of a 5 dpf *Tg(fli1a:nEGFP)*^{y7}*;Tg (lyve1b:DsRed)*^{nz101} transgenic and the lower panel is a lateral confocal fluorescent image of the same transgenic zebrafish as in the upper panel, where green blood vessel endothelial cells and red lymphatic vessels can be visualized. Human NPC cells could be xenotransplanted to the

NPC cells can then be monitored under confocal microscopy and the model can also be used for screening for potential anticancer therapies.

Immunotherapy for Nasopharyngeal Carcinoma

Immunotherapy: General Concepts

From the estimated 18 million new cases of cancer and ~9.6 million cancer mortalities reported for 2018 and with poor prognosis associated with many types of human cancers, this represents a growing global public health problem worldwide [6]. The primary reason for this is that the available standard of care for most cancers has not changed significantly, chemo and radiotherapy, with their associated side effects, and are still gold-standard treatments [104–106]. Moreover, these notable treatment approaches have showed significant improvements albeit only for specific types of cancer (i.e., hematological malignancies), but still remain very limited for many of the solid tumors [107, 108]. These constraints make necessary to develop new, better tolerated, less expensive, and more effective therapies, which could particularly help patients with advanced and metastatic diseases and improving not only their survival but also their quality of life. In this context, the accumulated knowledge regarding mechanisms involved in cancer immunity has resulted in the development of novel therapeutic approaches to treat this group of diseases. One of these new strategies is immunotherapy, essentially developed during the last two decades and consists in the modulation and stimulation of patients' own immune system against the tumor. This manipulation of patients' immunity involves different compartments of this homeostatic system such as immune-competent cells, receptors, and immune-mediator molecules among others. Recently, and after decades of treating cancer patients mostly by surgery and chemo- and radiotherapy, immunotherapy has emerged as a reliable therapeutic alternative for most patients [109].

Relevant Cancer Mouse Models for Preclinical Investigation of Immunotherapy

Despite the obvious genetic and immunological differences among species that constitute animal models, preclinical studies using these models are a necessary prerequisite for human application of new immunotherapies and useful tools for increasing our knowledge and understanding of mechanisms underlying cancer immunobiology. In this context, several mouse strains, both immunocompromised and immunocompetent, have been extensively used in order to validate new therapeutic strategies for further studies in human clinical trials. As already mentioned, the most commonly used experimental models are human xenograft cancer cells, obtained from immortalized cell lines or from patients' tumor biopsies, grown in immunodeficient mice such as severe combined immunodeficient (SCID) and athymic nude mice [110, 111]. On the other hand, the most extensively used immunocompetent mice strains are C57BL/6 and BALB/c and different modifications from their genetic backgrounds. Human xenograft in immunodeficient mice allows the evaluation of different specific immune-related mechanisms since many of these strains keep immunological functions partially (usually parts of the innate immune response). In addition, genetically engineered mouse models have been extremely useful in understanding oncogenesis as well as metastatic mechanisms by manipulating tumor suppressor genes and oncogenes [112, 113]. These models have been additionally improved by recent advances in genetic manipulation strategies such as RNA interference and gene editing (CRISPR-Cas9) technologies, for example a recent study reported the development of Cas9 mice to model lung cancer [114]. Another relevant experimental approach with animal models is the complete or partial reconstitution of human immune system by transplanting different human cell compartments in immunodeficient mice [115, 116]. These humanized mice models have additionally

allowed the evaluation of therapies in a human immunity context as a previous step before its approval for clinical trials.

Immunotherapeutic Approaches for Cancer Treatment

To date, it is well known that cancer patients can develop T-cells that are able to recognize and destroy tumor cells in vitro and in vivo [117]. Additionally, T-cells are able to proliferate in response to stimulation with autologous tumor cells by secreting cytokines such as interleukin-2 (IL-2), interferon gamma (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor-alpha (TNF-α) [118]. These observations have resulted in the identification and characterization of tumor antigens recognized by human T-cells [119, 120]. From these and other relevant findings, in the last two decades new therapies have been developed based on the modulation of cells and molecules from the immune system to induce *in vivo* antitumor responses [121, 122]. These immunotherapeutic approaches have included the use of the following: proinflammatory cytokines, fully humanized mAbs, cell-based immunotherapies, and several cancer vaccines [123–127]. Different cytokines such as IL-2, IFN-γ, and IFN-α have been used alone or in combination with chemotherapy with significant reduction of metastases, but without increasing the survival rates of treated patients and corresponding improvement in their quality of life [128–130]. Adoptive transfer of tumor-specific $CD8^+$ T-cells, with or without systemic immune suppression, has also been used in different clinical trials and although that data shows significant tumor reductions but this is associated with a high percentage of adverse reactions in treated patients [124, 125, 131]. Recently, T-cell molecular engineering modification with chimeric antigen receptors (CAR) to produce CAR-modified T-cells has substantially improved the clinical success of adoptive immunotherapy [132, 133]. More recently, new therapeutic targets have now been identified in cancer and immune cells, which have enabled the development of a wide range of mAbs aimed to modulate antitumor immune response and eradicate tumors [134, 135]. Indeed, the identification of immune check point molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), gave further knowledge for immune system manipulation. In this context, an anti-CTLA-4 monoclonal antibody (ipilimumab) was the first to achieve US Food and Drug Administration and European Medicine Agency approval, demonstrating statistical survival benefit in patients with melanoma [136]. Furthermore, preliminary clinical findings with blockers of additional immune-checkpoint proteins, such as programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1), have also shown important results in different settings of clinical trials, indicating broad and diverse opportunities to enhance antitumor immunity with the potential to produce durable clinical responses [137–139]. Remarkably, seminal studies which described these immunological mechanisms were recently recognized with the 2018 Nobel Prize in medicine and physiology for professors James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation."

Regarding cell-based immunization protocols, the use of dendritic cells (DCs), transfected with tumor-associated antigen (TAA) and immunomodulatory molecules-derived mRNA, or loaded with tumor cell lysates, among others, has been extensively explored. DC-based vaccination strategies are likely to be safe and capable of providing long-lasting protective immunity [140–143]. A DC-based vaccine, consisting in autologous monocyte-derived DCs loaded with an allogeneic heat shock-conditioned melanoma cell-derived lysate, has been included in a series of clinical trials in advanced malignant melanoma (MM) and prostate cancer patients. In this approach, lysate-loaded DCs can induce specific in vitro and in vivo antitumor and memory immune responses in patients with advanced MM with more than 60% classified as

immunological responder patients, showing a significant correlation with improved survival of stage IV MM patients compared with a median of 11 months for patients that were classified as non-responder [143]. Additionally, in prostate cancer patients this approach was capable of inducing memory T-lymphocytes, which might be associated with clinical responses, including decreased serum prostate specific antigen (PSA) levels and increased PSA doubling time [144]. However, despite the abilities of different immunotherapies for stimulating the antitumor response, an important percentage of patients remain refractory to these approaches. In fact, individual factors involved in the differential capacity of patients to respond to immunizations remain poorly understood.

Immunotherapy in NPC

As mentioned NPC is a subset of head and neck squamous cancers which remain with a poor outcome largely due to diagnosis at late stage of the disease and therapeutic options that advanced minimal compared to those for other cancers. Radiotherapy is broadly effective for NPC patients diagnosed at early stage or non-metastatic stage of disease progression. With the use of intensity-modulated radiation therapy (IMRT) that uses computer-controlled linear accelerators to deliver precise radiation doses to a malignant tumor or specific areas within the tumor, toxic side effects can be minimized to achieve good local control [145]. However, due to the low awareness of NPC as a disease and the associated symptoms often mistaken for common ailments, many patients upon diagnosis present with neck lumps in the clinic, indicating late stage disease and lymph node metastasis [146]. Treatment options for late stage NPC lesions include a combination of radiotherapy and cytotoxic chemotherapy, for example, cisplatin, 5-FU, paclitaxel, and gemcitabine [90]. For those patients with distant metastases, palliative platinum-based therapy followed by radiotherapy greater than 65-Gy at organs with metastatic lesions is followed [147]. While radiochemotherapy is curative for most patients, approximately 10% of the patients will experience local reoccurrence which further reduces a favorable outcome [146]. In this context, available targeted therapies, for example, cetuximab and nimotuzumab, anti-EGFR mAbs, have been explored but with mixed results [148, 149]. On the other hand, bevacizumab, a mAb, that targets VEGF has shown promise and when used with systemic therapy showed improved progression-free survival and improved quality of life of patients. However, together with the known toxicities associated with chemotherapy, side effects for bevacizumab include hypertension, bleeding, gastrointestinal perforation, cardiotoxicity, and thromboembolic events which may negate the favorable response [150]. Finally, surgery for metastatic NPC lesions but this option often depends on the proximity of tumor cells to vital blood vessel and nerve [151].

As mentioned above, a high percentage of treated cancer patients remain refractory to immune-related therapies, strongly suggesting that a combinatorial approach may be useful to improve the response rates of these patients. In this scenario, cancer vaccines, particularly dendritic cell (DC)-based vaccines, as well as check point inhibitors could be used as complementary treatments in cancer patients. Importantly, these approaches could be less toxic conferring an additional benefit for patients' quality of life. Nivolumab and pembrolizumab, two FDA-approved anti-PD-1 mAbs, constitute new therapeutic options for metastatic and chemotherapy-resistant NPC patients. A Phase II clinical trial (NCI-9742) investigated nivolumab in a small subset of patients with recurrent and metastatic NPC, and the data demonstrated a response rate of ~20% with minimal toxicity, suggesting further evaluation in larger cohorts of NPC patients [152]. Furthermore, data from the KRYNOTE-028 study (NCT02054806) investigated pembrolizumab in patients with PD-L1-positive advanced NPC. The data from this phase 1b clinical trial demonstrated a response rate of ~26% and the therapy was favorably tolerated [153]. Other clinical evaluations have explored the use of adoptive T-cell transfer as potential

Table 1 Current phase III clinical trials evaluating the efficacy of immunotherapy in combination with standard therapy for NPC

Clinical trial identifier	Interventions	Number of patients	Estimated completion date
NCT02578641	Gemcitabine, carboplatin, autologous EBV-specific cytotoxic lymphocytes	330 patients	December 2021
NCT02611960	Pembrolizumab	230 patients	March 2020
NCT03700476	Gemcitabine, cisplatin, IMRT, sintilimab as neoadjuvant	420 patients	January 2025
NCT03427827	Chemo-radiation followed by PD-1 antibody (SHR-1210) as adjuvant	400 patients	February 2024

treatment option for NPC patients. In a Phase II clinical trial, adoptive immunotherapy with EBV-specific cytotoxic T-lymphocytes (EBV-CTLs) was evaluated in combination with chemotherapy of gemcitabine and carboplatin, in patients with advanced EBV-positive NPC. The data obtained was favorable achieving a response rate of ~71% and 2- and 3-year overall survival rates of 62.9% and 37.1%, respectively. Importantly, a subgroup of patients were found to have either stable disease or with reduced tumor growth and overall the therapy was well tolerated, and common toxicities observed were fatigue and myalgia [154]. In a more recent Phase I/II clinical trial, efficacy of EBV-stimulated cytotoxic T-lymphocyte (EBV-CTL) immunotherapy was investigated in patients with active, recurrent, metastatic EBV-associated NPC. Although one patient demonstrated a complete response to this line of therapy, the overall response rate was low. The authors suggest that the poor response rate was likely due to differences in key parameters of the clinical trial, for example, requiring patients to have confirmed progressive disease as an inclusion criterion that would allow as an endpoint to distinguish responses to prior palliative chemotherapy from those to investigational immunotherapy. Also worth mentioning is that the authors noted that a subset of patients demonstrated robust responses to the same chemotherapy (docetaxel, gemcitabine) they had previously failed while receiving EBV-CTL immunotherapy. This likely suggests that immunotherapy was acting as a primer for enhancing patient's immune system to confer a greater renewed response to the chemotherapy [155]. Although the aforementioned studies demonstrated a broadly favorable response to the different available immunotherapies, these were undertaken in small cohorts of patients. As a consequent, there are several ongoing clinical trials that are investigating available immunotherapies in combination with standard therapy for NPC in much larger patient cohorts and these are detailed in Table 1. Expectations are that the data from these trials can pave the way for a new era of anticancer therapies that can improve prognosis and quality of life of NPC patients.

Concluding Remarks

While notable advances have been made in our understanding of NPC pathogenesis, the disease remains endemic in South East Asia with incidence rates and deaths likely to show annual increases. NPC still remains with a poor outcome, primarily due to salient symptoms, late detection, and limited therapeutic options. While several risk factors have been associated with NPC, it still remains unclear why the Chinese ethnicities in South East Asia succumb to this cancer. Other limitations include the lack of model systems and tools to mirror human NPC and the limited number of NPC cell lines that are currently available. Thus, a need is recognized for establishing clean and representative NPC cell lines for in-depth investigation on NPC with the goal of developing effective therapies for transition from *bench-to-bedside* for improving patient outcome. A need for large-scale population-based molecular epidemiologic

studies to elucidate how environmental, viral, and genetic factors interact in both the development and the prevention of NPC is also recognized. Finally, there is a united need for the NPC scientific community to work closely and collaborative to advance our current limited understanding of NPC pathogenesis.

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An In Vitro Model of Triple-Negative Breast Cancer

J. Russo and Y. Su

Abstract

We have characterized two highly tumorigenic and metastatic basal B TNBC cell lines, XtMCF and LmMCF, with the additional values of having the normal and early-stage counterparts of them. This model allows the study of the evolution of TNBC, and investigates molecular pathways at different stages of transformation and progression in a relatively constant genetic background. This constitutes an ideal model for developing targeted therapy in two important fields in cancer biology which are the epithelial mesenchymal transition (EMT) and cancer stem cells (CSC).

Keywords

17ß-estradiol · Human breast epithelial cells · Epithelial to mesenchymal transition · Triple-negative breast cancer · Chromatin remodeling · Breast epithelial cell transformation

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17ß-Estradiol Induces Transformation and Tumorigenesis in Human Breast Epithelial Cells

Breast cancer is a malignancy whose dependence on estrogen exposure has long been recognized, even though the mechanisms through which estrogens cause cancer are not clearly understood [1–17]. Our work was performed in order to determine whether 17ß-estradiol (E_2), the predominant circulating ovarian steroid, is carcinogenic in human breast epithelial cells and whether non-receptor mechanisms are involved in the initiation of breast cancer. For this purpose, the effect of four alternating 24 h treatment periods with 70 nM E_2 of the estrogen receptor alpha (ER-α) negative MCF-10F cell line on the in vitro expression of neoplastic transformation was evaluated [7, 8, 11] (Fig. 1).

E2-treatment induced the expression of anchorage-independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and loss of 9p11-13. Tumorigenesis in SCID mice was expressed only in invasive cells that in addition exhibited a deletion of 4p15.3-16. Tumors formed in SCID mice were poorly differentiated adenocarcinomas that were estrogen receptor α and progesterone receptor negative, expressed keratins, EMA, and e-cadherin. The relationship between cell motility in vitro and the ability of neoplastic cells to invade and metastasize in vivo is well known. This led us to evaluate the

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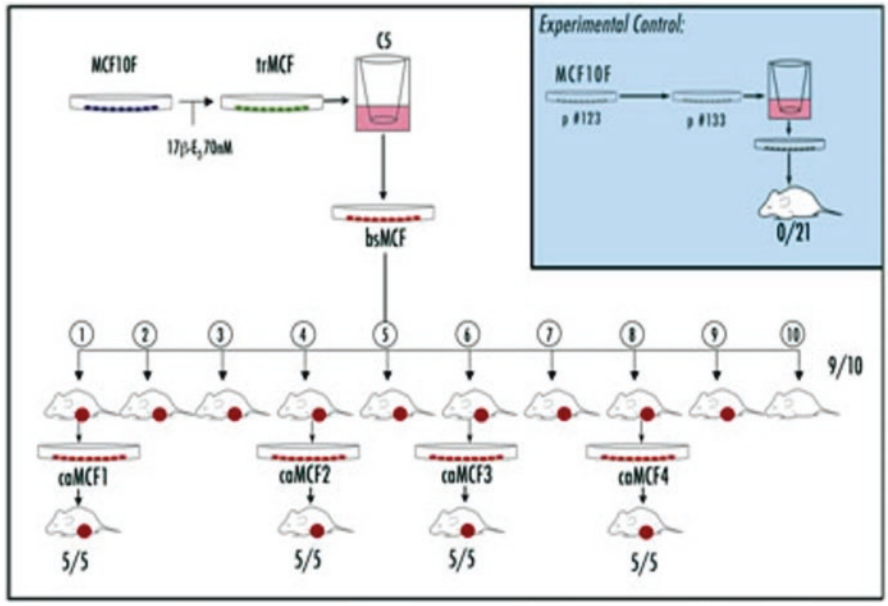

Fig. 1 Schematic representation of the experimental protocol. MCF-10F cells were treated with 70 nM 17ß-estradiol (17β-E_2 70 nM) for 24-h periods, twice a week during 2 weeks. After the last treatment the cells were passaged 7–9 times before being tested for colony efficiency, ductulogenesis in collagen, invasion in Matrigel and tumorigenesis in SCID mice. For the invasion assay, the cells were trypsinized and seeded in the invasion chambers at a concentration of 2.5×10^4 cells/well, incubated for 22 h and then the membranes of the inserts were cut and invasive cells (bsMCF) were cultured in 24-well plates. The invasive cells were expanded and evaluated for the expression of tumorigenesis in SCID mice. Nine out of ten mice injected with bsMCF developed tumors. Tumors from four of the animals were dissected in 0.5–1 mm size fragments, incubated in culture medium until confluent, generating cell lines from each tumor that were subsequently injected to five mice per cell line for the evaluation of their tumorigenic potential. All injected animals developed tumors. All tumors and cell lines were analyzed histopathologically and immunocytochemically as well as for fingerprint and CGH analyses. None of the animals injected with MCF-10F control cells or trMCF developed tumors

migratory behavior of the human breast epithelial cell line MCF-10F after neoplastic transformation with 70 nM of 17ß-estradiol (E_2), 4 OH estradiol (4-OH-E_2) and 2-OH estradiol (2-OH-E_2). Cells thus transformed express colony formation in agar methocel, loss of ductulogenic capacity in collagen matrix, and invasiveness in a Matrigel artificial membrane [7, 8, 11]. We set up a time-lapse video microscopy system to directly observe and capture the cells' images using a Nikon DXM digital camera attached to an Olympus IMT-2 microscope that was equipped with a Plexiglas incubation chamber. From each cell line, a random number of cells were selected for tracking at 1-hour intervals. Cell motility was evaluated by determining the speed of the tracked cell expressed in mm/min (**S**), the direction persistence in time (**P**), and the random motility coefficient (μ) that provides a measure of how fast a cell population will grow to cover a surface. Our findings [1] indicated that the transformation of HBEC by estrogen and its metabolites induces changes in cell motility in vitro, 4-OH-E_2 being the one inducing the most significant changes, its effect correlated to the expression of phenotypes indicative of cell transformation.

Epithelial to Mesenchymal Transition in Human Breast Epithelial Cells Transformed by 17-Beta-Estradiol

We have demonstrated that 17ß-estradiol (E_2) induces complete neoplastic transformation of the human breast epithelial cells MCF-10F [7, 8, 11]. E2-treatment of MCF-10F cells progressively induced high colony efficiency and

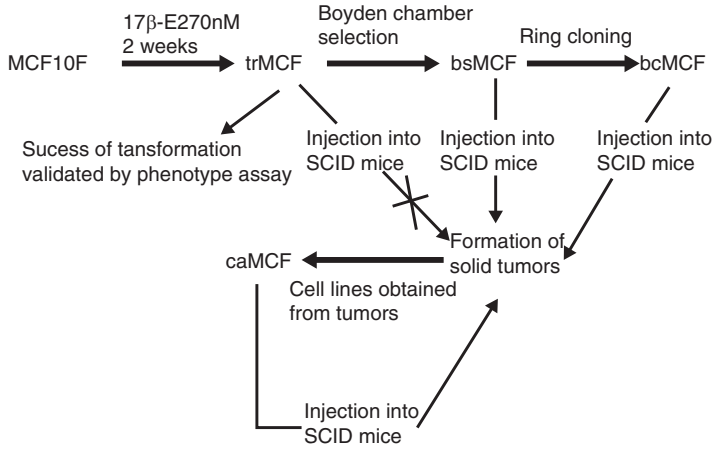

Fig. 2 Transformation of MCF-10F cells by 17ß-estradiol treatment. Experimental protocol: MCF-10F cells treated with 70 nM 17ß-estradiol (E_2) that expressed high colony efficiency (CE) and loss of ductulogenic capacity in collagen matrix were classified as transformed (trMCF). Transformed cells that were invasive in a Matrigel Boyden-type invasion chambers were selected (bsMCF) and plated at low density for cloning (bcMCF). MCF-10F, trMCF, bsMCF, and bcMCF were tested for carcinogenicity by injecting them into the mammary fat pad of 45-day-old female SCID mice. MCF-10F and trMCF cells did not induce tumors (canceled arrow); bsMCF and bcMCF formed solid tumors from which four cell lines, identified as caMCF, were derived and proven to be tumorigenic in SCID mice (reprinted from [11])

loss of ductulogenesis in early transformed (trMCF) cells, invasiveness in a Matrigel invasion chambers. The cells that crossed the chamber membrane were collected and identified as bsMCF, and their subclones designated bcMCF, and the cells harvested from carcinoma formation in SCID mice designated (caMCF) (Fig. 2) [11]. These phenotypes correlated with gene dysregulation during the progression of the transformation. The highest number of dysregulated genes was observed in caMCF cells, being slightly lower in bcMCF cells, and lowest in trMCF cells. This order was consistent with the extent of chromosome aberrations (caMCF > bcMCF >>> trMCF). Chromosomal amplifications were found in 1p36.12-pter, 5q21.1-qter, and 13q21.31-qter. Losses of the complete chromosome 4 and of 8p11.21-23.1 were found only in tumorigenic cells. In tumor-derived cell lines, additional losses were found in 3p12.1-14.1, 9p22.1-pter, and 18q11.21-qter [11].

Functional profiling of deregulated genes revealed progressive changes in the integrin signaling pathway, inhibition of apoptosis, acquisition of tumorigenic cell surface markers, and epithelial to mesenchymal transition. In tumorigenic cells, the levels of E-cadherin, EMA, and various keratins were low and CD44E/CD24 were negative, whereas SNAI2, vimentin, S100A4, FN1, HRAS and TGFβ1, and CD44H were high (Fig. 3) [11].

The phenotypic and genomic changes triggered by estrogen exposure that lead normal cells to tumorigenesis confirm the role of this steroid hormone in cancer initiation. Our work emphasizes the importance of being able to make a normal cell-like MCF-10F neoplastically transformed by a treatment with a natural hormone. More importantly the cell is estrogen receptor negative, indicating that the traditional pathway of action for estrogen and its receptors is not the main pathway of the neoplastic process. It is known that prolonged exposure to estrogen is a risk factor for human breast cancer, but the role of estrogen in the development of human breast cancer has been difficult to ascertain. There are three mechanisms that have been considered responsible for the carcinogenicity of estrogens: a receptor-mediated hormonal activity, cytochrome P450-mediated metabolic activation, and induction of aneuploidy. The

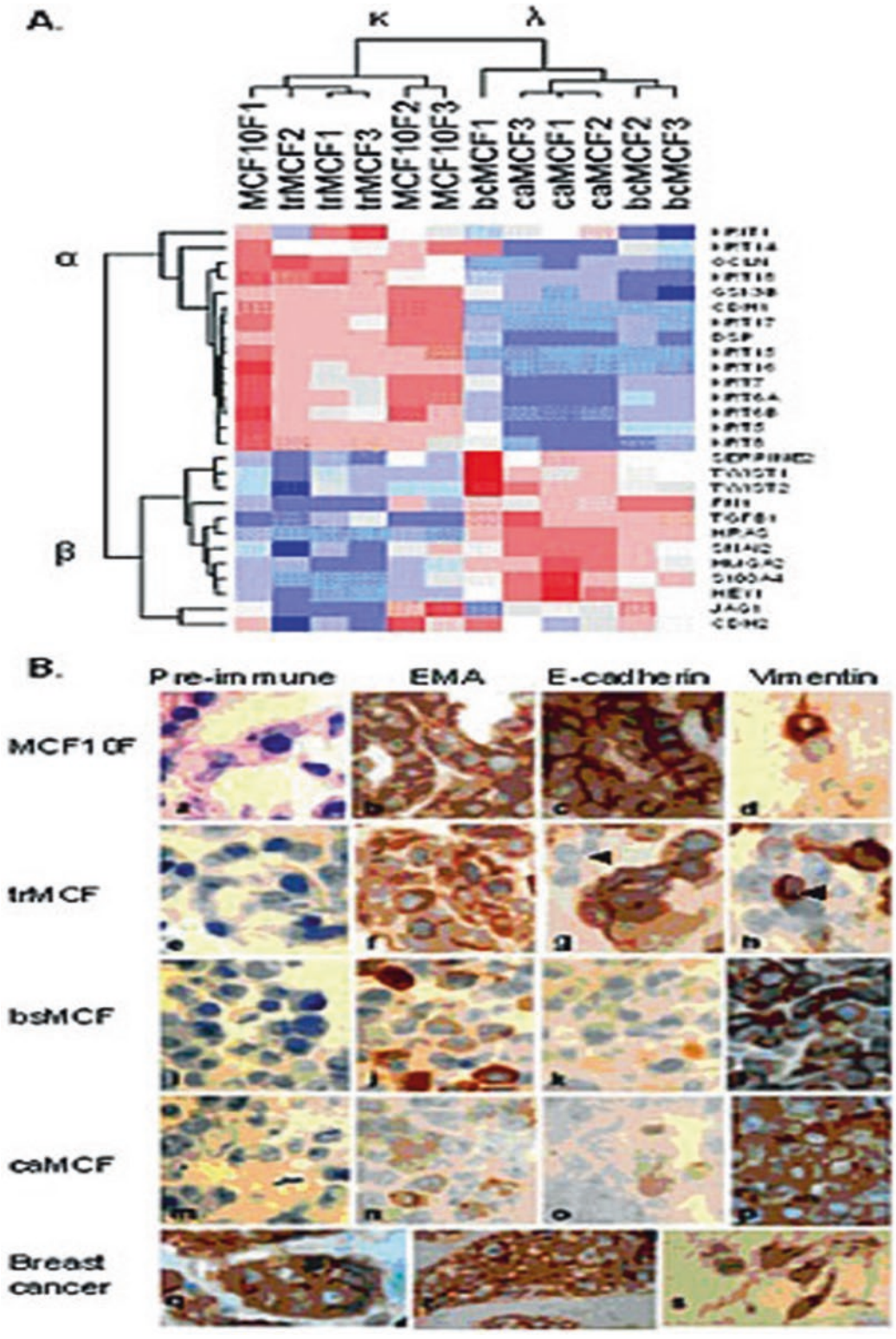

Fig. 3 (**a**) A list of EMT markers and promoting genes was generated a priory by literature search. Hierarchical clustering of cell lines and genes was performed using dChip software. Two sample clusters (κ and λ) and two gene clusters (α and β) were identified. The red, white, and blue colors represent level above, at, and below mean expression, respectively.

receptor-mediated hormonal activity of estrogen has generally been related to stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis. Since local synthesis of estrogen in the stromal component can increase the estrogen levels and growth rate of breast carcinoma, a paracrine mechanism is likely to account for interactions between aromatase-containing stromal cells and ER-containing breast tumor epithelial cells. More importantly, estrogen may not need to activate nuclear receptors alpha to initiate or promote breast carcinogenesis. We have evidence that ERP may also be involved in this process and that oxidative catabolism of estrogens mediated by various CYP complexes constitutes a pathway of their metabolic activation and generates reactive free radicals and intermediate metabolites reactive intermediates that can cause oxidative stress and genomic damage directly. Estrogen-induced genotoxic effects include increased mutation rates, MSI, and LOH in chromosomes 3 and 11. Compromised DNA repair system allows accumulation of genomic lesions essential to estrogen-induced tumorigenesis. Metabolic biotransformation of estrogen does occur in human mammary explant culture. Increased formation of catechol estrogens as a result of elevated hydroxylation of 17β-estradiol at C-4 and C-16a positions has been observed in human breast cancer patients and in women at a higher risk of developing this disease. There is also evidence that formation of superoxide and hydrogen peroxide, as a result of the metabolism of estrogen, might also be involved in estrogen-mediated oxidative stress. In fact, a substantial increase in base lesions observed in the DNA of invasive ductal carcinoma of the breast has been postulated to result from the oxidative stress associated with metabolism of 17β-estradiol. Altogether the data thus far accumulated indicate that more than one pathway may be necessary to initiate neoplastic transformation and maintaining of the transformation phenotypes leading to tumorigenesis.

Developing a Unique Model of Triple Negative Breast Cancer

Triple-negative breast cancer (TNBC) represents a heterogeneous group of cancers characterized by a lack of ER, PgR, and HER2 expression. Cluster analysis of human TNBC identified six subtypes displaying unique gene expression and ontologies [18]. Approximately 80% of TNBC show features of basal-like cancers [19]. Transcriptional profile analysis assigned 21 TNBC cell lines into three clusters: luminal, basal A, and basal B [20–22]. Basal A contains cell lines such as BT-20, Sum149, and MDA-MB-468, which preferentially express genes such as *CK5/6*, *CK14*, and *EGFR*. Basal B includes cell lines such as MDA-MB-231, Sum159pt, and Hs578t, which preferentially express genes such as *CD44*, *VIM*, and *SNAI2*, and exhibits a stem-cell-like profile [20]. This classification of TNBC cell lines is closely associated with cell morphology and invasive potential. Basal B cells have a more mesenchymal-like appearance and are less differentiated and much more invasive compared to the other two clusters. Analysis of the relationship between TNBC cell

Fig. 3 (coninued) (**b**) Detection of epithelial and mesenchymal markers by immunocytochemistry: (*a*) Histological sections of MCF-10F cells, reacted with pre-immune mouse serum, were used as the negative control (×100); (*b*) MCF-10F reacted for EMA (×100); (*c*) MCF-10F reacted for E-Cadherin (×100); (*d*) MCF-10F reacted for vimentin (×100); (*e*) trMCF cells reacted with pre-immune mouse serum used as negative control (×100); (*f*, *g*, and *h*) trMCF cells reacted for EMA, E-cadherin, and vimentin, respectively (×100); (*i*) bsMCF cells reacted with pre-immune mouse serum as a negative control (×100); (*j*, *k*, *l*) bsMCF cells reacted for EMA, E-cadherin, and vimentin, respectively (×100); (*m*) caMCF tumor cell line cells reacted with pre-immune mouse serum used as negative control (×100); (*n*, *o*, *p*) caMCF tumor cell lines reacted for EMA, E-cadherin, and vimentin, respectively (×100); (*q* and *r*) invasive ductal carcinoma of the breast as positive control and immunoreacted for EMA and E-cadherin, respectively (×100); (*s*) histological section of an invasive adenocarcinoma immunoreacted for vimentin (×100) (Reprinted from: [11])

lines and tumor subtypes showed that most of basal A and basal B cell lines resemble basal-like tumors [20], indicating that TNBC cell lines are suitable for investigations of subtype-specific cancer cell biology.

Although there are over 20 commercially available TNBC cell lines, MDA-MB-231 is the most widely used in vitro and in vivo. In BALB/CAJCI-nu/nu mice, it took 5 weeks to form a xenograft around 6.5 mm in diameter with the subcutaneous injection of 5×10^6 MDA-MB-231 cells [23]. MDA-MB-468 cells had a growth speed similar to MDA-MB-231 in the same mouse strain [23].

The growth speed of MDA-MB-231 xenograft in CB17/SCID was almost the same as in nude mice, while BT-549 cells grew a little bit slower than MDA-MB-231 cells in CB17/SCID mice [24]. Sum149 and Sum159 are two highly tumorigenic cell lines; it was reported that the injection of 1×10^5 cells in nonobese diabetic SCID mice could produce tumors in 3/4 and 5/6 mice, respectively [25]. But these two cell lines are mainly used for the study of inflammatory breast cancer [26, 27].

We have established [28] a progressive TNBC model (Fig. 4) consisting of normal MCF-10F, transformed cell line trMCF, and tumorigenic cell lines bsMCF, XtMCF, and LmMCF. Compared to the other nine tumorigenic TNBC cell lines, our cell lines XtMCF and LmMCF are the most tumorigenic and metastatic.

The expression of cytokeratin 18 (CK18) confirmed the epithelial origin of this cell model, and we observed that CK18 was down-regulated in bsMCF cell line and its derivatives. Furthermore, CK18 was lost in the lung metastases, whereas still present in the xenografts of both XtMCF and LmMCF cells, suggesting down-regulation of CK18 may be related to breast tumor progression [29]. Our study also showed that CK5-positive cell number was inversely correlated to clinical stage of TNBC [30, 31], suggesting that our cell model reflects features of TNBC progression.

The EMT process is not only closely related to cancer invasion and metastasis but also conferred to the generation of cancer stem cells (CSC) [32–34]. As bsMCF-luc, XtMCF, and LmMCF have undergone EMT, we evaluated

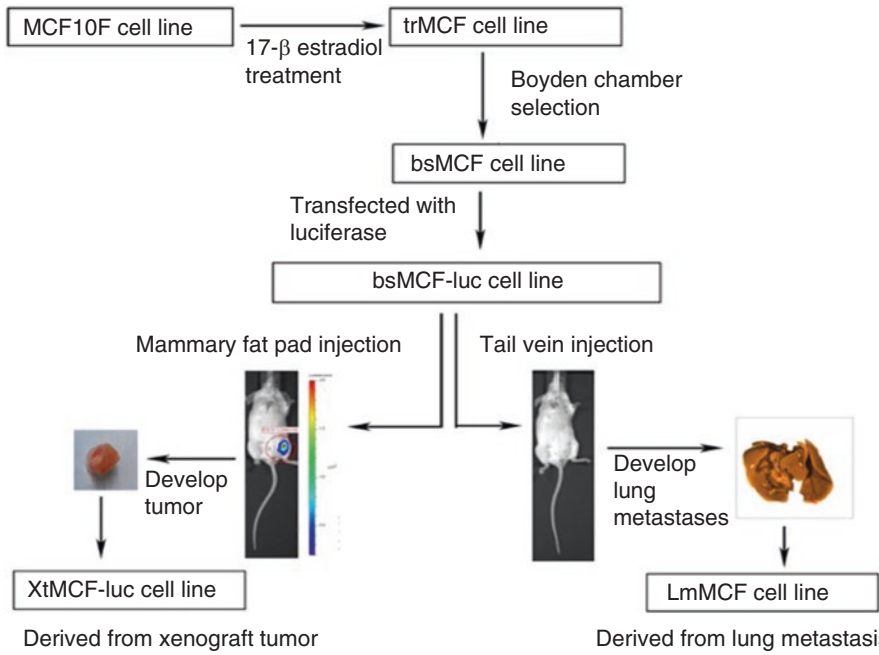

Fig. 4 Schematic representation of the establishment of a TNBC model

their CSC properties and the results showed that they could form tumorspheres, and the number of tumorspheres was progressively increasing from bsMCF-luc to XtMCF and LmMCF cells, consistent with in vivo tumorigenic and metastatic potential.

In this work [30], we postulated that the evaluation of CSC markers would give us a rationale for the high tumorigenic and metastatic potential of these two cell lines (Figs. 5 and 6). Our results showed that the bsMCF-luc and XtMCF cells were $CD24^{low}/CD44^{+}$, whereas LmMCF cells were $CD44^{+}$ with moderate CD24 expression. $CD24^{-/low}/CD44^{+}$ has been frequently used as CSC markers of breast cancers [35–37]. However, it was shown that the percentage of $CD24^{-/low}/CD44^{+}$

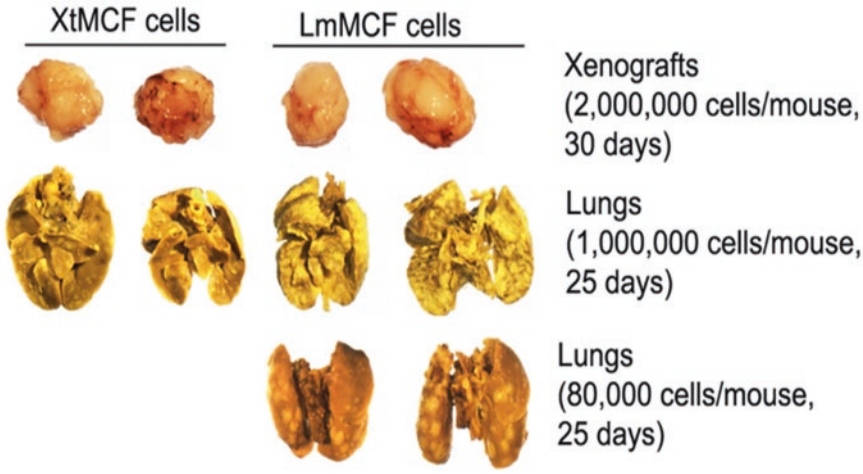

Fig. 5 XtMCF and LmMCF cells display high tumorigenic and metastatic potential. Representative pictures of xenografts and lungs fixed with Bouin's solution. Magnification: 6.3× for xenografts, 8× for lungs (Reprinted from: [28])

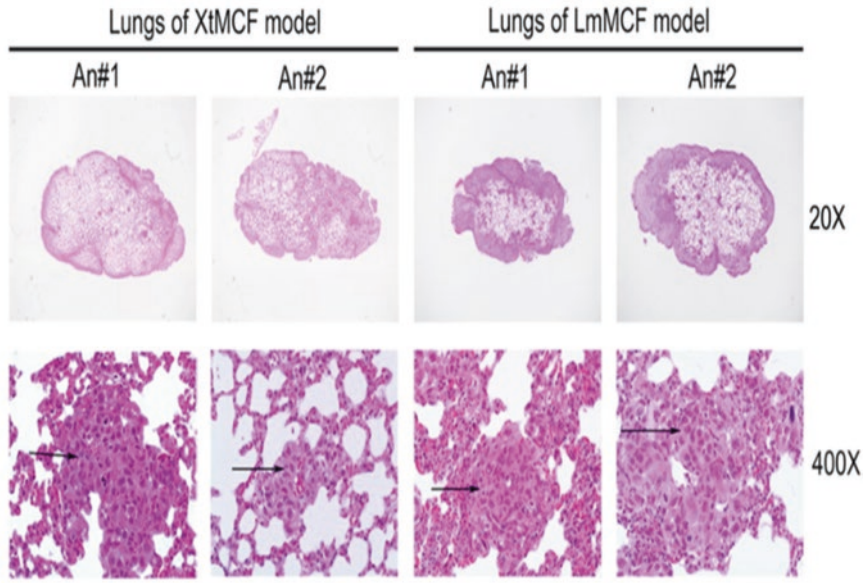

Fig. 6 H&E staining of lungs from the injection of 1×10^6 cells into tail vein. LmMCF cells are more metastatic than XtMCF cells. Arrows indicate the metastases. Magnifications are shown in figure (Reprinted from: [28])

associates with a basal-like phenotype, not tumorigenicity, but $CD24^{-/low}/CD44^{+}/EpCAM^{+}$ cells enrich for tumorigenicity [31, 36]. EpCAM induces expressions of reprogramming factors and EMT genes, regulates EMT progression, and tumorigenesis [38]. In addition, EpCAM can be cleaved at several sites, and the nuclear translocation of cytoplasmic domain (EpCID) associates with Wnt pathway and promotes cell proliferation and tumor formation in mice [39]. One of the EpCAM cleavage sites between two arginine residues (AA80 and AA81) was detected and described in the late 1980s, but the functional consequence is still unknown [40]. Interestingly, we observed the expression of EpCAM in the cell lines we examined by immunofluorescence staining and WB, but the EGF-like domain of EpCAM was absent in mesenchymal-like cells, suggesting the EGF-like domain might be cleaved off from the cleavage site between AA80 and AA81. This was supported by other workers [41–47]. The majority of commercial antibodies for EpCAM react with overlapped or partly overlapped epitope at EGF-like domain [48]. This may result in failing detection of EpCAM in cells which have undergone EMT. Our study indicates that the EGF-like-domain-cleaved-off EpCAM may be associated with the EMT process. Furthermore, although the total level of EpCAM is low in mesenchymal-like cells, the subcellular localization of EpCAM may be more important to the EpCAM nuclear indicating a strong activation of Wnt signaling in these cells.

Chromatin Remodeling During Human Breast Epithelial Cell Transformation

We have shown that treatment of the human breast epithelial cells MCF-10F with 17β-estradiol (E2) induces transformation and tumorigenesis. DNA amounts and chromatin supraorganization change in E2-transformed MCF cells [49]. Feulgen-DNA content and chromatin supraorganization were involved during E2-induced transformation and tumorigenesis of the MCF-10F cells. Image analysis was performed for non-transformed and E2-transformed MCF cells, highly invasive cells (C5), and for cell lines (C5-A6-T6 and C5-A8-T8) derived from tumors generated by injection of C5 cells in SCID mice (Fig. 1). A decrease in Feulgen-DNA amounts and nuclear sizes induced by E2 treatment was accented with selection of the highly invasive tumorigenesis potential. However, in the tumor-derived cells, a high variability in cellular phenotypes resulted inclusive in near-polyploidy. Significant changes in textural parameters, including nuclear entropy, indicated chromatin structural remodeling with advancing tumorigenesis. An increased variability in the degree of chromatin packing states in the E2-transformed MCF cells was followed by reduction in chromatin condensation and in contrast between condensed and non-condensed chromatin in the highly invasive C5 cells and tumor-derived cell lines. These observations confirmed previous data [11], showing the role of chromatin remodeling and epigenetic control in the transformation of human breast epithelial cells. We found that a network of several signaling pathways affecting the expression and/or function of a complex hierarchical network of transcription factors (TFs) has been partially elaborated. Known signaling pathways include multiple tyrosine kinase receptors leading to Ras-mediated activation of MAPK and PI3K pathways, TGF-β, Notch, and Wnt. Evidence for enhanced TGF-β and Wnt signaling pathways was found in the EMT expressing bcMCF and caMCF cells. TGF-β acting through Smad transcriptional complexes can repress expression of the Id TFs (Id1, Id2, Id3) and activate HMGA2, a DNA binding protein important for chromatin architecture. Expression of HMGA2 is known to regulate several EMT controlling TFs including TWIST1, SNAI1, and SNAI2 (Slug) (Fig. 7). TGF-β and Wnt signaling also affect the expression of several additional EMT-regulating TFs including ZEB1 (TCF8), TCF3 (E2A encoding E12 and E47), and LEF1. Analysis of the EMT expressing bcMCF cell line revealed the absence of expression of the secreted frizzled-related protein 1 (SFRP1), a repressor of Wnt signaling. One allele of SFRP1 was deleted in these cells, with the remaining apparently silenced by methylation, accounting for the 28-fold reduction of this transcript. Loss

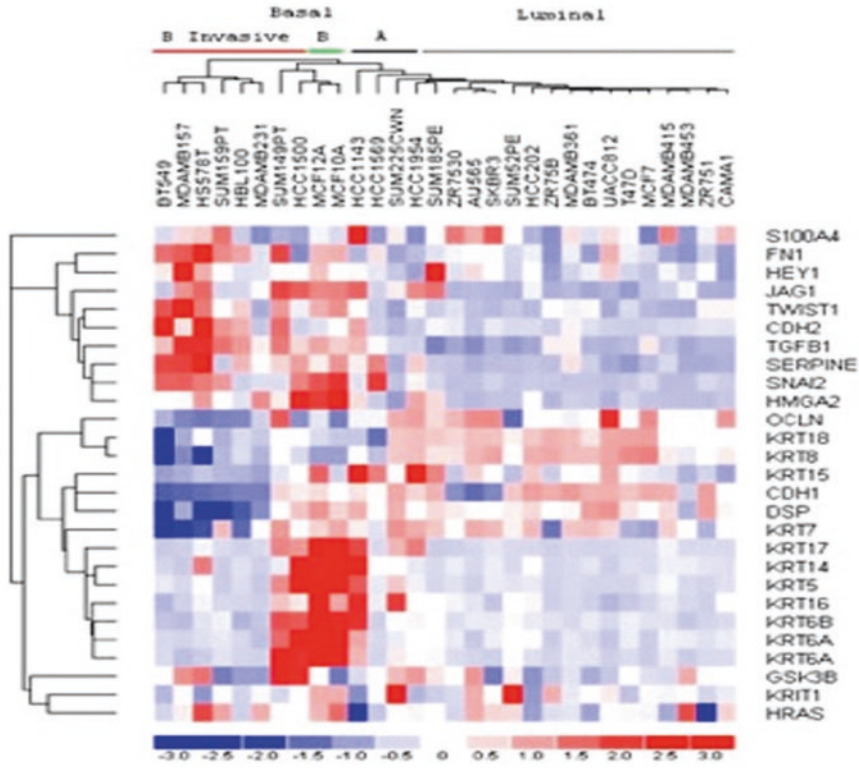

Fig. 7 Heat map showing the EMT (Reprinted from: [11])

and epigenetic inactivation of SFRP1 occurs often in invasive breast cancer and is associated with poor prognosis. Inspection of the SFRP1 expression levels in Basal B cell lines showed absent calls for four of the eight invasive cell lines, and eightfold decreases in another three invasive cell lines relative to the non-invasive MCF-10A cells (data not shown). Inspection of the expression files for bcMCF cells and the eight invasive Basal B cell lines revealed that LEF1 was always absent, while TCF 3 and TCF 8 were expressed.

As we have described above the TNBC cell consisting of normal like breast epithelial cell line MCF10F, the trMCF cell line that was transformed from MCF10F cells, the tumorigenic bsMCF cell line derived from trMCF, and two highly tumorigenic and metastatic cell lines XtMCF and LmMCF established from bsMCF cells have undergone epithelial mesenchymal transition (EMT), exhibiting a mesenchymal-like feature, providing a good cell model for identifying new treatments for TNBC.

Concluding Remarks

The relevance of this work is the development and characterization of two highly tumorigenic and metastatic basal B TNBC cell lines, XtMCF and LmMCF. To the best of our knowledge, they are the most tumorigenic and metastatic TNBC cell lines compared to all reported cell models used for TNBC studies. In addition, the normal and early-stage counterparts of these two cell lines are also available. Altogether, these cell lines can be used to study the evolution of TNBC, investigate molecular pathways at different stages of transformation and progression in a relatively constant genetic background, and most importantly, identify new treatments for TNBC. In addition, XtMCF and LmMCF cell lines present CSC properties and can be used for developing CSC-targeted therapy. The finding that the EGF-like domain of EpCAM is cleaved off in cancer cells which have undergone EMT also provides

new insights in the research of EMT and CSC, two important fields in cancer biology.

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Emerging Role of Novel Biomarkers of Ly6 Gene Family in Pan Cancer

Geeta Upadhyay

Abstract

Stem cell antigen-1 (Sca-1) is the first identified member of mouse Ly6 gene family. We discovered that Sca-1 disrupts TGFβ signaling and enhances mammary tumorigenesis in a DMBA-induced mammary tumor model. Sca-1 gene is lost during evolution in humans. Human Ly6 genes Ly6D, LyE, LyH, and LyK on human chromosome 8q24.3 genes are syntenic to the mouse chromosome 15 where Sca-1 is located. We found that Ly6D, E, H, and K are upregulated in human cancer compared to normal tissue and that the increased expression of these genes are associated with poor prognosis of multiple types of human cancer. Several other groups have indicated increased expression of Ly6 genes in human cancer. Here we described the relevance of expression of human Ly6D, LyE, LyH, and LyK in functioning of normal tissues and tumor progression.

Keywords

Biomarkers · Ly6 · TGF-beta · Survival outcome

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Introduction

Stem cell antigen-1 (Sca-1) was the first identified member of mouse Ly6 gene family [1]. Sca-1 has been described as marker of tissue-resident stem cells and also been recognized as cancer initiating cell population in multiple mouse model of mammary, prostate, and lung cancer among other cancer types [2–4]. We described that Sca-1 binds with TGFβ receptor 1 (TβR1) and disrupts the TGFβ receptor complex, leading to tumorigenic progression in mouse model of mammary tumorigenesis [5]. A direct homologue of Sca-1 is missing in humans; however, multiple members of mouse Ly6 genes and syntenic human Ly6 genes have been described. Human Ly6 gene family usually contain LU domain. These molecules are scattered throughout the human chromosome 1, 2, 6, 8, 11, and 19 [6]. We will concern our description to human Ly6D, LyE, LyH, and Ly6K, which are present on human chromosome 8q24.3.

Expression of Ly6D, E, H, and K Genes in Normal Tissues

Ly6D

Ly6D RNA is expressed in highest quantities in esophageal tissue and in skin as shown in HPA dataset, GTEx dataset, and Fantome5 datasets as

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visualized in Human Protein Atlas available from www.proteinatlas.org [7–9]. The RNA expression was undetectable or expressed at a very low level in normal ovary, lung, testis, colon, breast, and prostate (Fig. 1a–c). The protein expression corroborated with RNA expression, showing increased expression of Ly6D in esophageal tissue and in skin (Fig. 2).

Ly6E

Ly6E RNA expression was identified in liver tissue, placenta, lung, and spleen by three different databases (Fig. 3a–c) as visualized in Human Protein Atlas available from www.proteinatlas.org [7–9]. The corroborative protein data was not yet available at the human protein atlas. Lowest expression of Ly6E RNA in normal tissues was found in pancreas and skeleton tissues (Fig. 3a–c). The Ly6E expression is restricted to syncytiotrophoblast cells of the mouse placenta [10].

Ly6H

Ly6H RNA expression was identified highest in Brain tissue in HPA dataset, GTEx dataset, and Fantome5 (Fig. 4a–c) as visualized in Human Protein Atlas available from www.proteinatlas.org [7–9]. Ly6H plays an important role in glutamatergic signaling in brain [11].

Ly6K

Ly6K RNA is expressed highest in testis in HPA dataset, GTEx dataset, and Fantome5 datasets (Fig. 5a–c) as visualized in Human Protein Atlas available from www.proteinatlas.org [7–9]. The RNA expression was undetectable or expressed at a very low level in normal ovary, lung, testis, colon, breast, and prostate. The protein expression data showed that Ly6K is exclusively expressed in testis (Fig. 6). Male Ly6K homozygous knockout mice showed normal mating habits; however, they were infertile. Female Ly6K homozygous knockout mice were fertile. The male infertility was found to be associated with sperm migration [12].

Expression of Ly6D, E, H, and K Genes in Tumor Tissues

Ly6D

We looked for RNA expression of Ly6D in various publically available dataset and visualized using Oncomine [13] and TCGA [14] as described by Luo et al. [15]. Ly6D was found to be increased in tumors of ovarian, colorectal, gastric, breast, lung, bladder, brain and CNS, cervical, esophageal, head and neck and pancreatic cancer compared to normal tissue in multiple studies. The increased expression of Ly6D was associated with poor survival in ovarian, colorectal, gastric, breast and lung cancer [15] (Table 1). Since our publication new clinical data were available and added to KM plotter tool [16] (http: //kmplot.com/), which showed that increased Ly6D expression is associated with poor prognosis in renal clear cell carcinoma and pancreatic ductal adenocarcinoma (Fig. 7). Recently Ly6D expression in addition to OLFM4, S100A7 was found to be associated with distant metastasis of estrogen receptor-positive breast cancer [17]. Ly6D was shown to be increased in aggressive forms of head and neck cancer [18].

Ly6E

We looked for RNA expression of Ly6E in various publically available dataset and visualized using Oncomine [13] and TCGA [14] as described by Luo et al. [15]. Ly6E was found be increased in tumors of ovarian, colorectal, gastric, breast, lung, bladder, brain and CNS, cervical, esophageal, head and neck and pancreatic cancer compared to normal tissue in multiple studies. The increased expression of Ly6E was

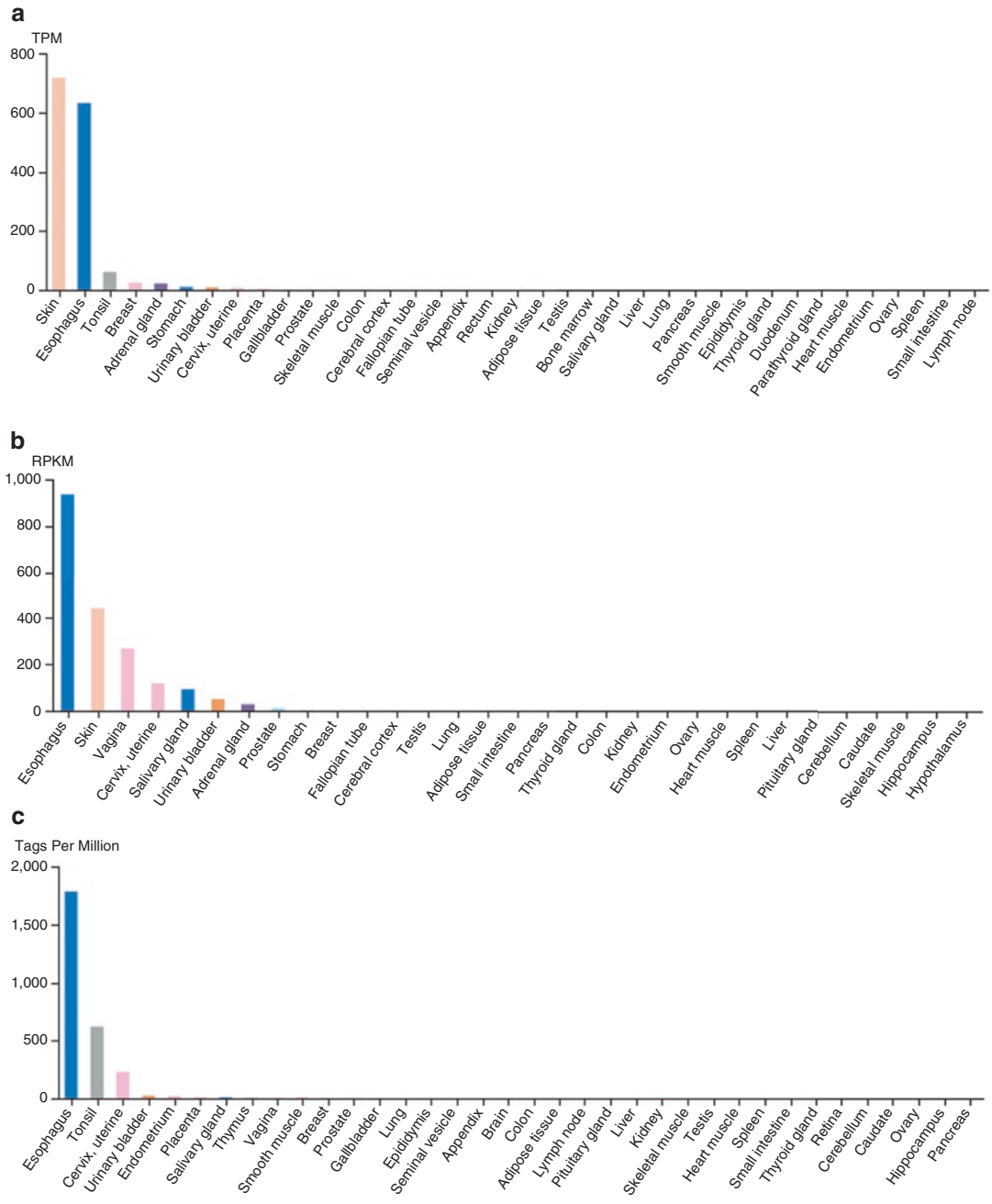

Fig. 1 RNA expression of Ly6D in normal human tissues. (**a**) HPA data set, (**b**) GTEx data set, and (**c**) FANTOM dataset. The data was visualized on the human proteome webtool with expression filter on. X-axis shows the name of tissue type used. Y-axis shows the units of RNA expression as described below. HPA dataset: RNA-seq tissue data is reported as mean TPM (transcripts per million), corresponding to mean values of the different individual samples from each tissue. Color coding is based on tissue groups, each consisting of tissues with functional features in common. GTEx dataset: RNA-seq data is reported as median RPKM (reads per kilobase per million mapped reads), generated by the Genotype-Tissue Expression (GTEx) project

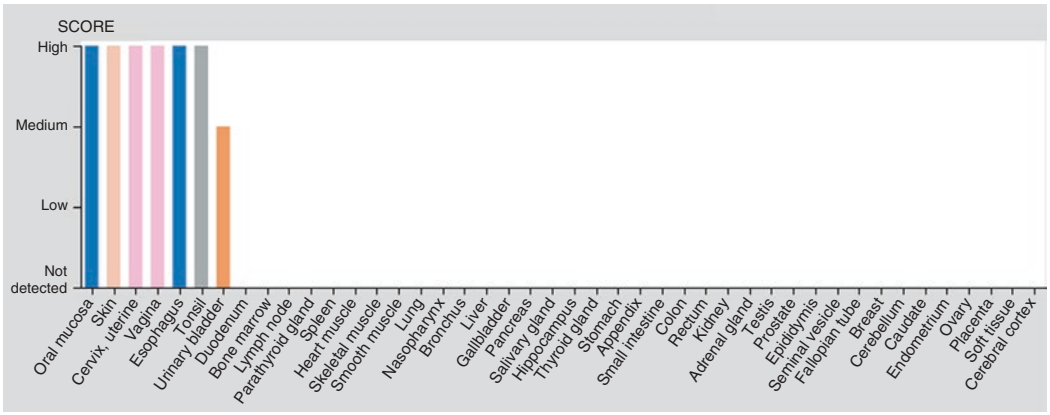

Fig. 2 Ly6D protein expression in normal tissues. Immunohistochemistry was performed using anti-Ly6D antibody-rabbit polyclonal affinity purified antibody HPA024755, HPA064317 from Sigma-Aldrich as described in the human protein atlas webtool: https://www.proteinatlas.org/ENSG00000167656-LY6D/tissue

associated with poor survival in ovarian, colorectal, gastric, breast, lung, bladder, and brain and CNS cancer patients [15] (Table 1). Recent data added to KM plotter [16] (http://kmplot.com/) show that increased expression of Ly6E is associated with poor overall survival of renal papillary cell carcinoma and good prognostic marker for renal clear cell carcinoma (Fig. 8a, b). These new data indicated that increased expression of Ly6E is associated with poor overall survival of pancreatic ductal adenocarcinoma (Fig. 8c). The use of genome wide data analysis has prompted several new reports showing increased expression of Ly6E in bladder cancer and gastric cancer [19, 20]. The Ly6E gene has been also associated with more aggressive stem-like cells in hepatocellular carcinoma, pancreatic carcinoma, colon, and kidney [21–23].

Ly6H

We looked for RNA expression of Ly6H in various publically available dataset and visualized using Oncomine [13] and TCGA [14] as described by Luo et al. [15]. Ly6H was found be increased in tumors of ovarian, colorectal, gastric, breast, lung, bladder, brain and CNS, cervical, esophageal, head and neck and pancreatic cancer compared to normal tissue in multiple studies. The increased expression of Ly6H was associated with poor survival in ovarian, colorectal, gastric, and breast cancer patients [15] (Table 1). Recent data added to KM plotter [16] (http://kmplot.com/) show that increased expression of Ly6H is associated with poor overall survival of renal clear cell carcinoma and pancreatic ductal adenocarcinoma (Fig. 9a, b).

Ly6K

We looked for RNA expression of Ly6K in various publically available dataset and visualized using Oncomine [13] and TCGA [14] as described by Luo et al. [15]. Ly6K was found be increased in tumors of ovarian, colorectal, gastric, breast, lung, bladder, brain and CNS, cervical, esophageal, head and neck and pancreatic cancer compared to normal tissue in multiple studies. The increased expression of Ly6K was associated with poor survival in ovarian, colorectal, gastric, breast, lung, bladder, and brain and CNS cancer patients [15] (Table 1). Increased expression of Ly6K in metastatic ER-positive breast cancer [24, 25], esophageal squamous cancer [26], gingivobuccal cancers [27], bladder cancer [28], and lung cancer [29] was observed. Recent data added to KM plotter [16] (http://kmplot.com/) show that increased expression of Ly6K is associated with poor overall survival of renal clear cell carcinoma, renal papillary cell carcinoma, and uterine corpus endometrial carcinoma (Fig. 10a–c).

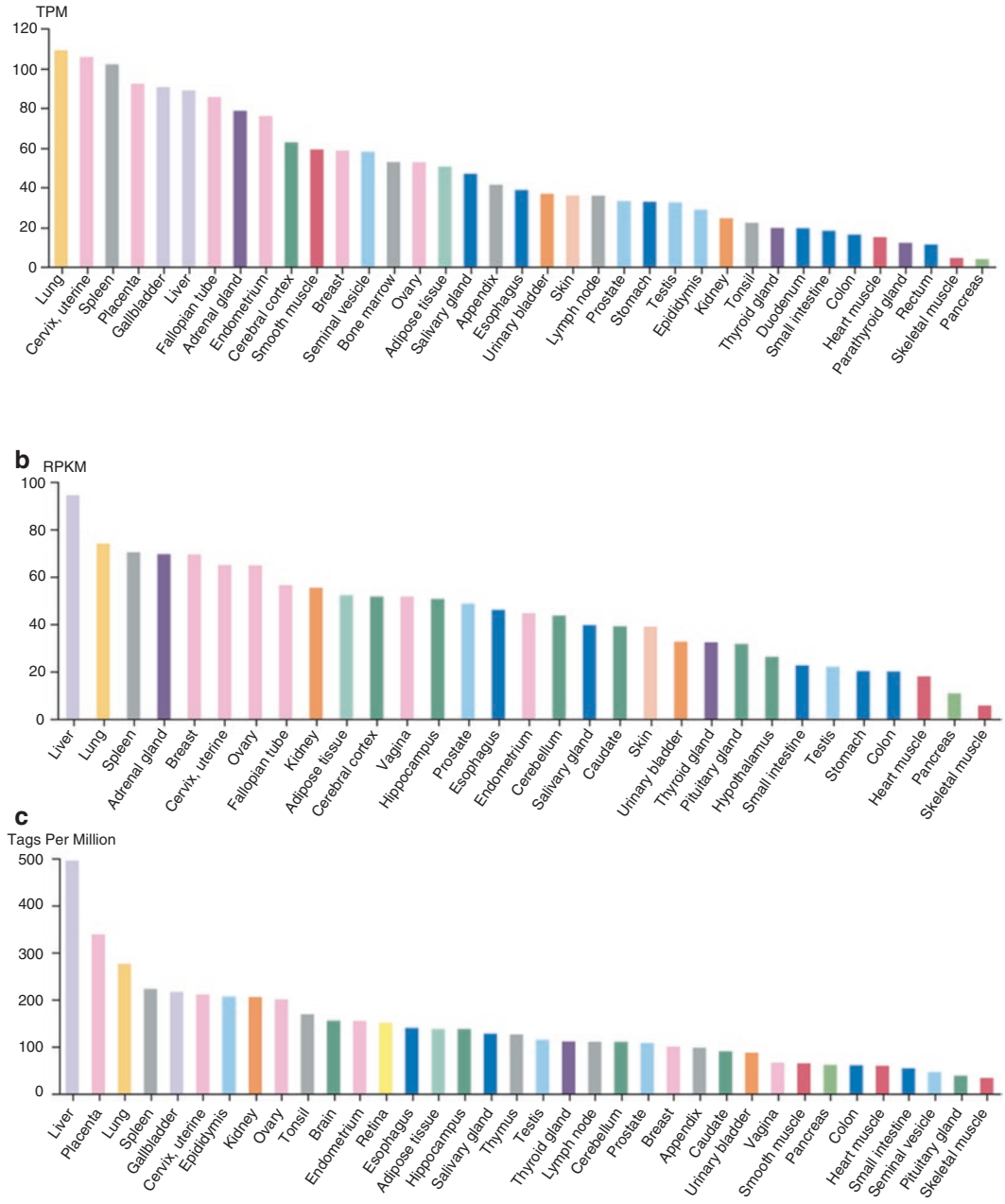

Fig. 3 RNA expression of Ly6E in normal human tissues. (**a**) HPA data set, (**b**) GTEx data set, and (**c**) FANTOM dataset. The data was visualized on the human proteome webtool with expression filter on. X-axis shows the name of tissue type used. Y-axis shows the units of RNA expression as described below. HPA dataset: RNA-seq tissue data is reported as mean TPM (transcripts per million), corresponding to mean values of the different individual samples from each tissue. Color-coding is based on tissue groups, each consisting of tissues with functional features in common. GTEx dataset: RNA-seq data is reported as median RPKM (reads per kilobase per million mapped reads), generated by the Genotype-Tissue Expression (GTEx) project

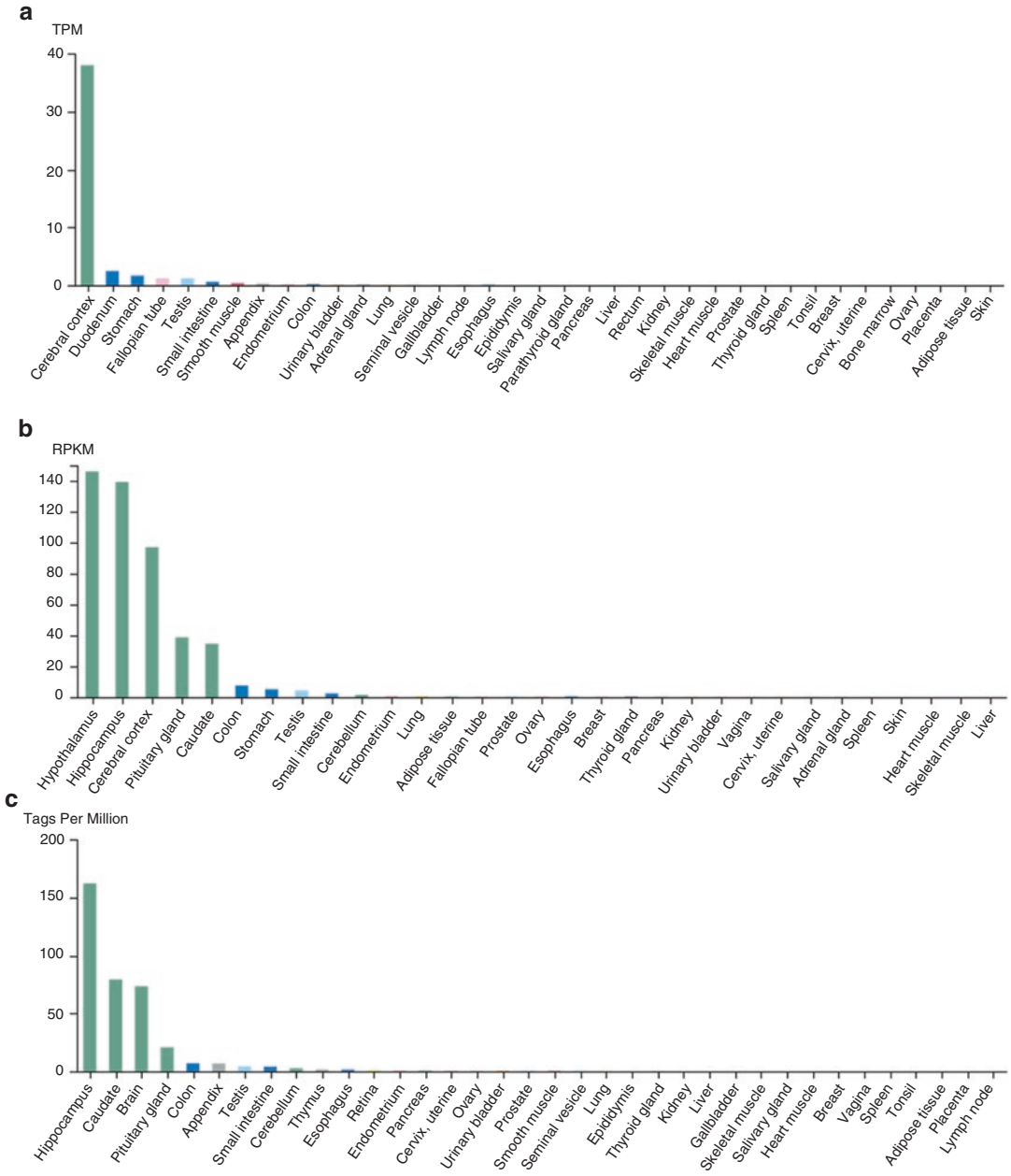

Fig. 4 RNA expression of Ly6H in normal human tissues. (**a**) HPA data set, (**b**) GTEx data set, and (**c**) FANTOM dataset. The data was visualized on the human proteome webtool with expression filter on. X-axis shows the name of tissue type used. Y-axis shows the units of RNA expression as described below. HPA dataset: RNA-seq tissue data is reported as mean TPM (transcripts per million), corresponding to mean values of the different individual samples from each tissue. Color coding is based on tissue groups, each consisting of tissues with functional features in common. GTEx dataset: RNA-seq data is reported as median RPKM (reads per kilobase per million mapped reads), generated by the Genotype-Tissue Expression (GTEx) project

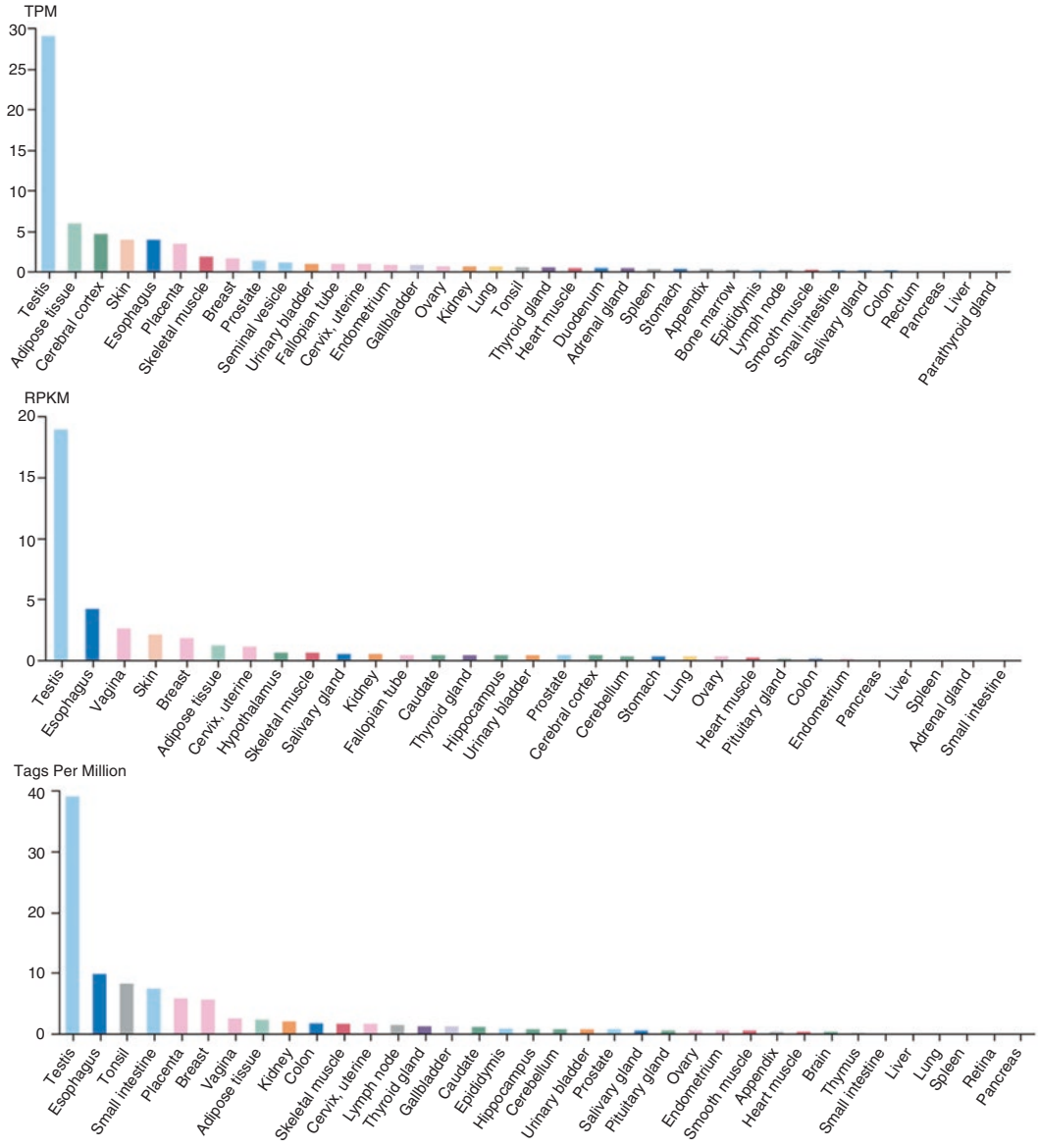

Fig. 5 RNA expression of Ly6K in normal human tissues. (**a**) HPA data set, (**b**) GTEx data set, and (**c**) FANTOM dataset were visualized on the human proteome webtool with expression filter on. X-axis shows the name of tissue type used. Y-axis shows the units of RNA expression as described below. HPA dataset: RNA-seq tissue data is reported as mean TPM (transcripts per million), corresponding to mean values of the different individual samples from each tissue. Color coding is based on tissue groups, each consisting of tissues with functional features in common. GTEx dataset: RNA-seq data is reported as median RPKM (reads per kilobase per million mapped reads), generated by the Genotype-Tissue Expression (GTEx) project

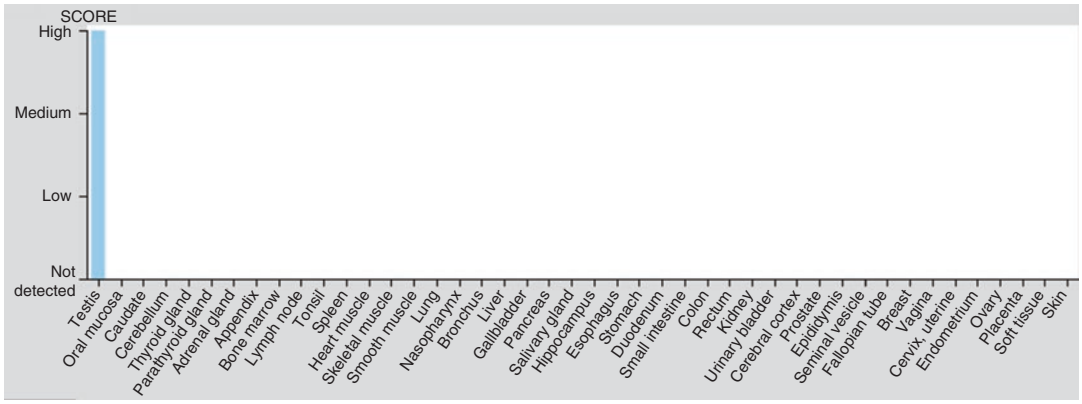

Fig. 6 Ly6K protein expression data in normal tissues. Ly6K protein is exclusively expressed on human testis. Immunohistochemistry was performed using anti-Ly6K antibody-rabbit polyclonal affinity-purified antibody HPA017770 and protein A/G purified mouse monoclonal antibody AMAb90986, AMAb90987 from Sigma-Aldrich as described in the human protein atlas webtool: https://www.proteinatlas.org/ENSG00000160886-LY6K/tissue

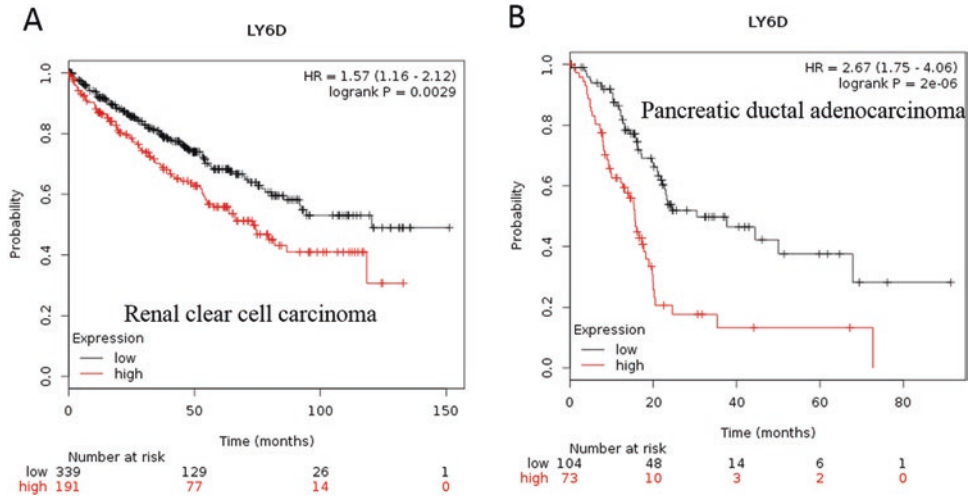

Fig. 7 Increased Ly6D mRNA expression in cancer and patient survival. High Ly6D RNA expression leads to poor survival in (**a**) renal clear cell carcinoma and (**b**) pancreatic ductal adenocarcinoma

Table 1 Correlation of high mRNA expression and patient survival outcome in multiple cancer types

Cancer type	Genes	Expression in tumors ($p < 0.05$)	Survival analysis ($p < 0.05$)
Ovarian	LY6D	Up	Poor prognosis
	LY6E	Up	Poor prognosis
	LY6H	Up	Poor prognosis
	LY6K	Up	Poor prognosis
Colorectal	LY6D	Up	Poor prognosis
	LY6E	Up	Poor prognosis
	LY6H	Up	Poor prognosis
	LY6K	Up	Poor prognosis
Gastric	LY6D	Up	Poor prognosis
	LY6E	Up	Poor prognosis
	LY6H	Up	Poor prognosis
	LY6K	Up	Poor prognosis
Breast	LY6D	Up	Poor prognosis
	LY6E	Up	Poor prognosis
	LY6H	Up	Poor prognosis
	LY6K	Up	Poor prognosis
Lung	LY6D	Up	Poor prognosis
	LY6E	Up	Poor prognosis
	LY6H	Up	OS (NS), others (NA)
	LY6K	Up	Poor prognosis
Bladder	LY6D	Up	OS (NS), others (NA)
	LY6E	Up	Poor prognosis
	LY6H	NS	OS (NS), others (NA)
	LY6K	Up	Poor prognosis
Brain and CNS	LY6D	Up	OS (NS), others (NA)
	LY6E	Up	Poor prognosis
	LY6H	Up	OS (NS), others (NA)
	LY6K	Up	Poor prognosis
Cervical	LY6D	Up	OS (NA), RFS (NS)
	LY6E	Up	OS (NA), RFS (NS)
	LY6H	Up	OS (NA), RFS (NS)
	LY6K	Up	OS (NA), RFS (NS)
Esophageal	LY6D	Up	OS (NS), others (NA)
	LY6E	Up	OS (NS), others (NA)
	LY6H	Up	OS (NS), others (NA)
	LY6K	Up	OS (NS), others (NA)
Head and neck	LY6D	Up	OS (NS), others (NA)
	LY6E	Up	OS (NS), others (NA)
	LY6H	Up	OS (NS), others (NA)
	LY6K	Up	OS (NS), others (NA)
Pancreatic	LY6D	Up	OS (NS), others (NA)
	LY6E	Up	OS (NS), others (NA)
	LY6H	Up	OS (NS), others (NA)
	LY6K	Up	OS (NS), others (NA)

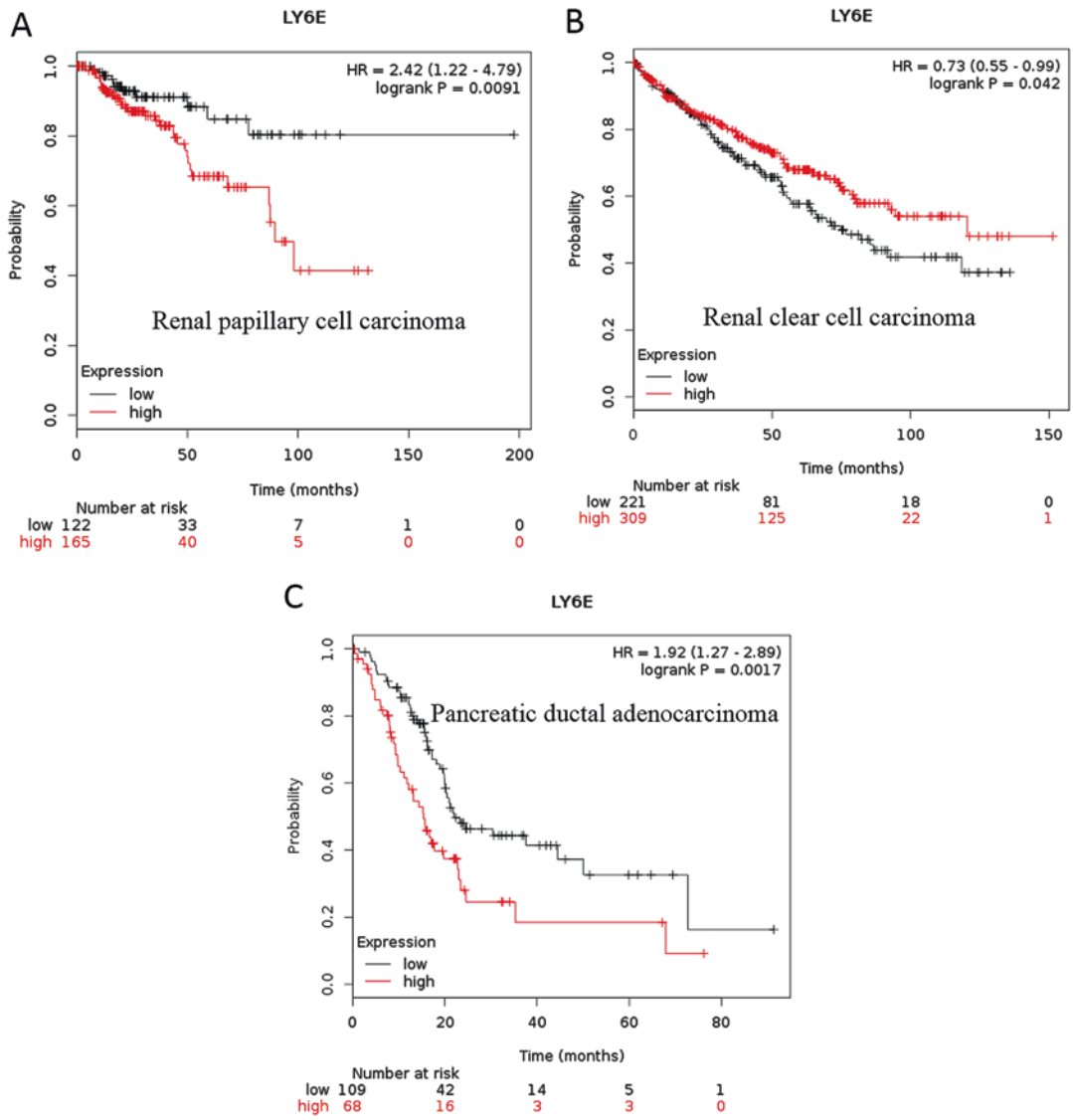

Fig. 8 Increased Ly6E mRNA expression in cancer and patient survival. High Ly6E RNA expression leads to poor survival in (**a**) renal papillary cell carcinoma, good prognosis for (**b**) renal clear cell carcinoma, poor survival in (**c**) pancreatic ductal adenocarcinoma

Mechanisms Associated Ly6D, E, H, and K Gene Family

Ly6D

The functional role of Ly6D in human or mouse is not very well described. Ly6D was found to be important in selection of CD4+CD8+ subset of T cells in thymus [30]. Ly6D was shown to be involved in cell adhesion using NIH3T3 fibroblast cells [31]. The molecular pathways which may lead to regulation of Ly6D remain to be understood.

Ly6E

We discovered that Ly6E is required for increased TGFβ signaling, IFNγ/PDL1 signaling in breast cancer [32]. Ly6E was also shown to modulate

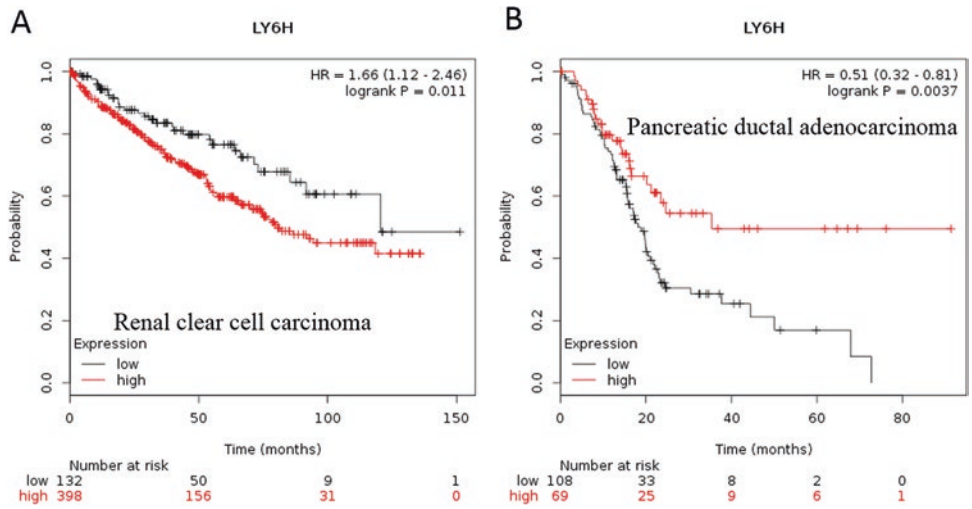

Fig. 9 Increased Ly6H mRNA expression in cancer and patient survival. High Ly6H RNA expression leads to poor survival in (**a**) renal clear cell carcinoma and (**b**) pancreatic ductal adenocarcinoma

the PTEN/PI3K/Akt/HIF-1 axis in breast cancer cell lines [33]. Ly6E was found to be important in enhancing the infectivity of multiple, enveloped RNA viruses in late steps using influenza A virus model [34]. Ly6E has been shown to be an interferon-inducible gene in lymphoid cells and plays an important role in HIV infection [35, 36]. Mouse Ly6E is a receptor syncytin A and plays important role in syncytiotrophoblast fusion and placental morphogenesis [37, 38].

Ly6H

Ly6H is required for nicotine-induced glutamatergic signaling via synaptic signaling of alpha7 nicotinic acetylcholine receptors [11].

Ly6K

We discovered that Ly6E is required for increased TGFβ signaling, IFNγ/PDL1 signaling in breast cancer [32]. High Ly6K expression is shown to have been linked with low expression of tumor suppression microRNA 500a-3p in non-small cell lung carcinoma [39]. AP1 activation is shown to be important in increased expression of Ly6K in breast cancer cell lines [40]. Ly6K protein expression in testis was shown to be stabilized by TEX101, a glycoprotein important in fertility [41].

Summary

The important role of Ly6 gene family in physiology and disease is emerging. The Ly6 gene family members can emerge as valid therapeutic target in correcting conditions of infection, nicotine addiction, cancer, immunotherapy, and fertility. Our laboratory is focused on understanding the molecular mechanism of Ly6 protein in cancer and validating Ly6 molecules as therapeutic targets in targeted cancer therapy. The network analysis of Ly6 gene family members showed that Ly6 signaling is involved in a broad range of molecules including growth factor, nuclear receptor, and micro RNAs (Fig. 11a). Pathway studio network analysis showed that Ly6 gene family affect multitude of cellular fate and cell–cell interaction with microenvironment ranging from growth, apoptosis, autophagy, and immune response (Fig. 11b).

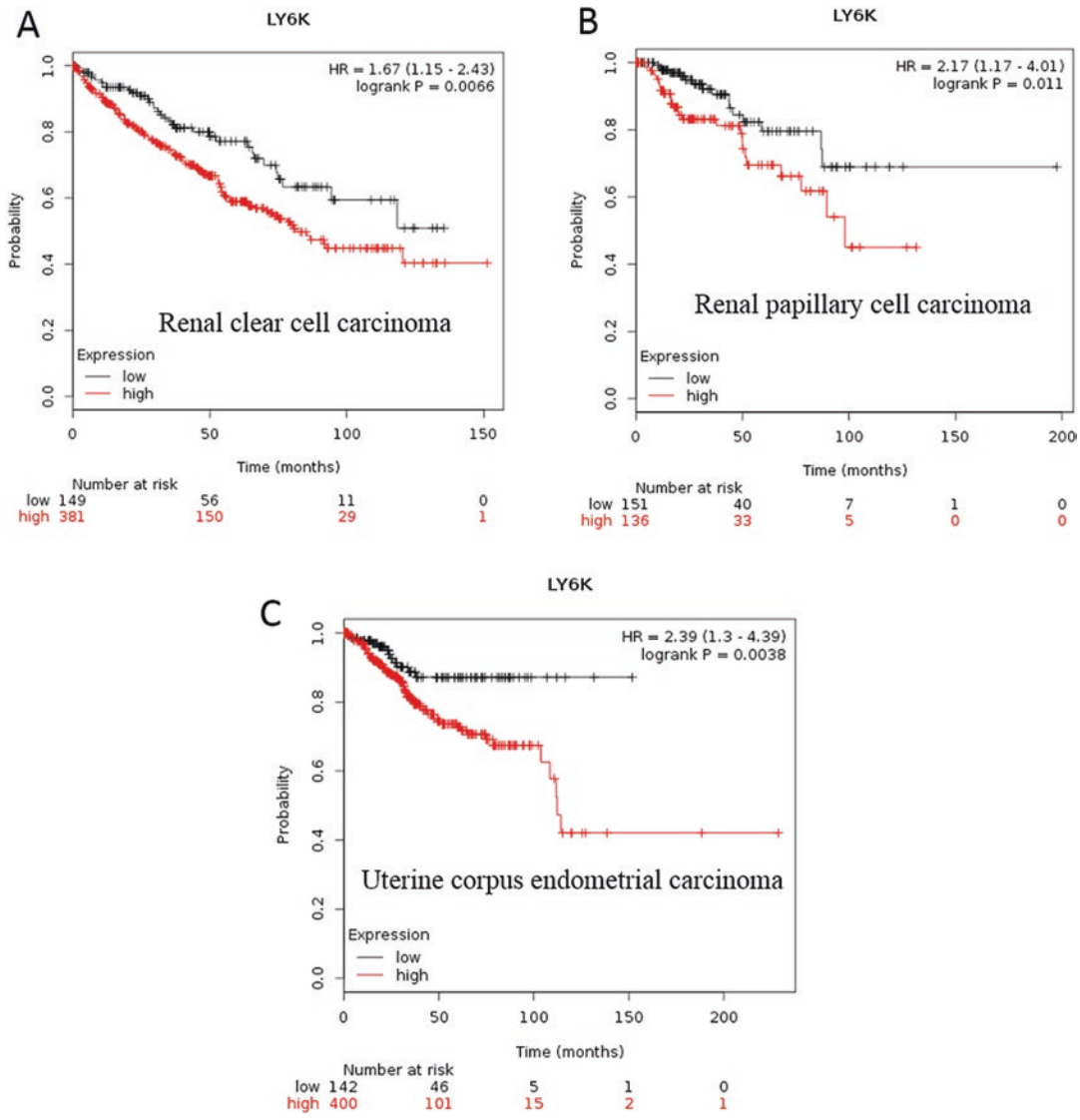

Fig. 10 Increased Ly6K mRNA expression in cancer and patient survival. High Ly6K RNA expression leads to poor survival in (**a**) renal clear cell carcinoma, (**b**) renal papillary cell carcinoma, and (**c**) pancreatic ductal adenocarcinoma

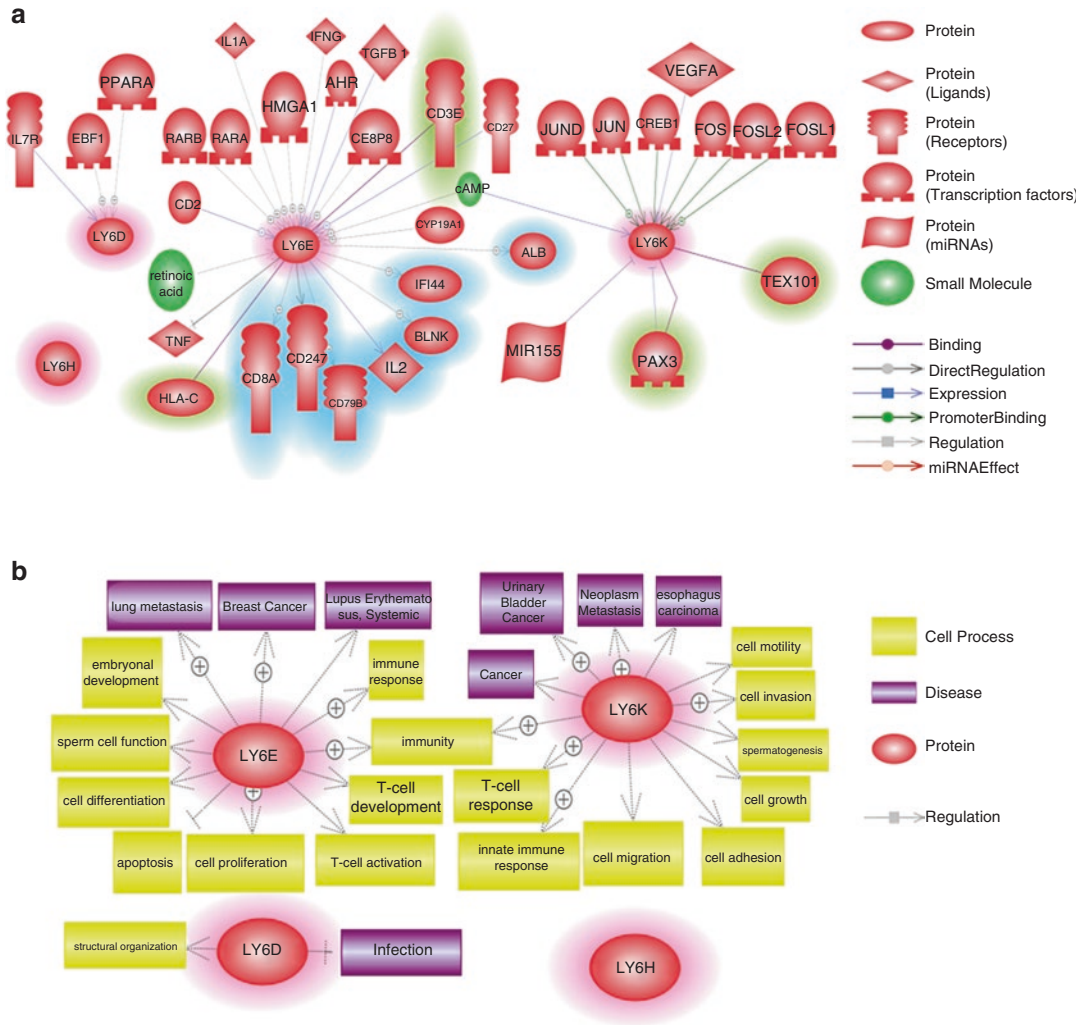

Fig. 11 Network analysis of Ly6 gene family members. (**a**) Pathway studio network analysis showed that Ly6 signaling is involved in broad range of molecules including growth factor, nuclear receptor, and micro RNAs. The upstream regulators are not highlighted, the downstream effectors are highlighted with blue, and the potential binding partners are highlighted with green. (**b**) Pathway studio network analysis showed that Ly6 gene family affect multitude of cellular fate and cell–cell interaction with microenvironment ranging from growth, apoptosis, autophagy, and immune response

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The Oncoprotein Gankyrin/PSMD10 as a Target of Cancer Therapy

Jun Fujita and Toshiharu Sakurai

Abstract

Gankyrin (also called PSMD10, p28, or $p28^{GANK}$) is a crucial oncoprotein that is upregulated in various cancers and assumed to play pivotal roles in the initiation and progression of tumors. Although the in vitro function of gankyrin is relatively well characterized, its role in vivo remains to be elucidated. We have investigated the function of gankyrin in vivo by producing mice with liver parenchymal cell-specific gankyrin ablation (*Alb-Cre;gankyrin$^{f/f}$*) and gankyrin deletion both in liver parenchymal and in non-parenchymal cells (*Mx1-Cre;gankyrin$^{f/f}$*). Gankyrin deficiency both in non-parenchymal cells and parenchymal cells, but not in parenchymal cells alone, reduced STAT3 activity, interleukin-6 production, and cancer stem cell marker expression, leading to attenuated tumorigenic potential in the diethylnitrosamine hepatocarcinogenesis model. Essentially similar results were obtained by analyzing mice with intestinal epithelial cell-specific gankyrin ablation (*Villin-Cre;Gankyrin$^{f/f}$*) and gankyrin deletion both in myeloid and epithelial cells (*Mx1-Cre;Gankyrin$^{f/f}$*) in the colitis-associated cancer model. Clinically, gankyrin expression in the tumor microenvironment was negatively correlated with progression-free survival in patients undergoing treatment with Sorafenib for hepatocellular carcinomas. These findings indicate important roles played by gankyrin in non-parenchymal cells as well as parenchymal cells in the pathogenesis of liver cancers and colorectal cancers, and suggest that by acting both on cancer cells and on the tumor microenvironment, anti-gankyrin agents would be promising as therapeutic and preventive strategies against various cancers, and that an in vitro cell culture models that incorporate the effects of non-parenchymal cells and gankyrin would be useful for the study of human cell transformation.

Keywords

Gankyrin · Proteasome · Tumor suppressor · Gene therapy · Tumor microenvironment

Liver cancer is the sixth most common cancer overall (0.78 million cases, 5.6%) but ranks second as cause of death (0.74 million, 9.1%) [1]. Around 80% of liver cancer in adults is hepatocellular carcinoma (HCC), and HCC is often diagnosed at advanced stages when most curative therapies are of limited efficacy. Furthermore, HCC is resistant

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to conventional chemotherapy and rarely amenable to radiotherapy, leaving HCC with a very poor prognosis [2]. Although a causal relationship between chronic damage, inflammation, and carcinogenesis has been widely recognized, the exact molecular mechanism of hepatocarcinogenesis remains to be elucidated. In 2000, we discovered gankyrin as an oncoprotein overexpressed in 100% of HCCs analyzed [3]. Further studies have suggested that gankyrin is a promising molecular target for diagnosis, treatment, and prevention of almost all types of cancers besides HCC (reviewed in references [4–9]).

Isolation of Gankyrin from Hepatocellular Carcinoma

Gankyrin (Gann ankyrin-repeat protein; "Gann" in Japanese means cancer) was originally identified as an oncogene product consistently overexpressed in HCCs [3]. Independently, it was purified as the p28 component [10] or a protein bound to the S6b subunit of the 19S regulator of the 26S proteasome [11]. Thus gankyrin is also known as PSMD10 (proteasome 26S subunit, non-ATPase 10), although subsequent studies have demonstrated that gankyrin transiently binds to the 26S proteasome and works as a chaperone for the assembly of the 19S regulator [12]. Gankyrin is a small 25kD cytoplasm–nucleus shuttling protein, and highly conserved throughout evolution (~40% identity to yeast Nas6p). Structurally, gankyrin consists of seven ankyrin repeats [13]. Ankyrin repeat is a functional domain involved in protein–protein interactions.

Enhanced Degradation of RB (Retinoblastoma-Associated Protein) and p53 (Cellular Tumor Antigen p53) by Gankyrin

Gankyrin plays a key role in regulating the cell cycle [14]. Gankyrin contains the RB-recognition motif LxCxE in the C-terminal domain, and binds RB in vitro and in vivo [3]. Forced expression of gankyrin in immortalized mouse fibroblasts and human tumor cells confers growth in soft agar and tumor formation in nude mouse. Gankyrin deactivates the RB tumor suppressor pathway at multiple levels (Fig. 1a) [3]. Gankyrin binds to CDK4, competing with and displacing p16INK4A and p18INK4C, inhibitors of cyclin-dependent kinases, which results in active CDK4, hyperphosphorylation of RB, and release of the E2F transcription factor to activate DNA synthesis genes. Binding of gankyrin also increases the rate of RB ubiquitylation and degradation by the proteasome.

When overexpressed, gankyrin inhibits apoptosis of cells that have been exposed to DNA-damaging agents [15]. This anti-apoptotic activity is due, at least partly, to increased degradation of p53. Gankyrin binds to the E3 ubiquitin ligase MDM2 in vitro and in vivo, increasing the ubiquitylation and subsequent proteasomal degradation of p53, resulting in the reduced transcription of p53-dependent pro-apoptotic genes [15]. The fact that gankyrin simultaneously binds the proteasomal S6b ATPase and RB [16] suggests that gankyrin could be a carrier of ubiquitylated proteins to the 19S regulator of the 26S proteasome to enhance their degradation.

Gankyrin as a Killer of Multiple Tumor Suppressor Proteins

In addition to the two major tumor suppressors RB and p53, gankyrin binds to other tumor suppressor proteins such as C/EBPα [17], TSC2 [18], HNF4α [19], and CUGBP1 [20], and enhances their ubiquitylation and subsequent degradation by the proteasome (Fig. 1b). Gankyrin inhibits p16 [3], PTEN [21], and FIH-1 (factor inhibiting HIF-1) [22] as well.

NF-κB/RelA is a transcription factor that is hyperactivated in many types of cancers and leads to inhibition of apoptosis. Interestingly, many studies have shown that inhibition of NF-κB in hepatocytes enhances hepatocarcinogenesis [23]. Gankyrin directly binds to NF-κB/RelA and suppresses its activity by modulating acetylation via SIRT1 [24], exporting RelA from

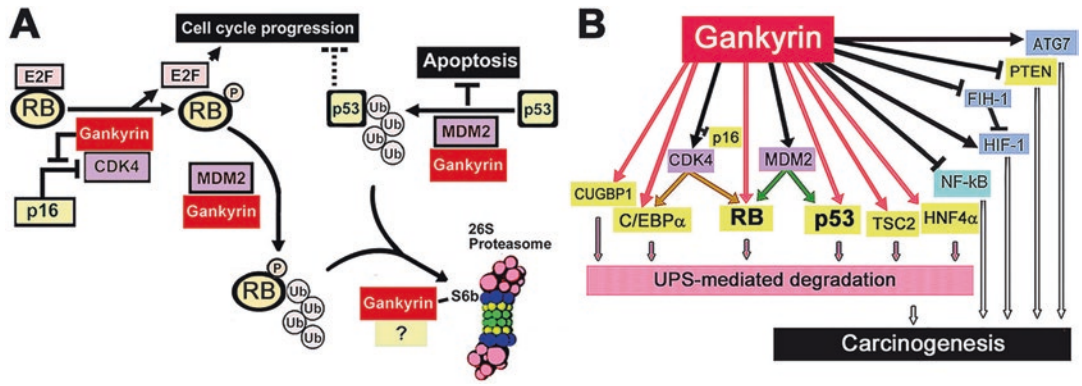

Fig. 1 Interaction of gankyrin with many proteins. (**a**) Activities of gankyrin on cell cycle control and apoptosis. In the presence of gankyrin, CDK4 is protected from the inhibitory effect of INKs (p16 and p18). Thus, RB is hyperphosphorylated and degraded, whereas E2F transcription factors are released to trigger expression of DNA synthesis genes. More p53 is ubiquitylated by gankyrin-bound MDM2 and degraded to suppress p53-dependent apoptosis and cell cycle arrest. *P* phosphate, *Ub* ubiquitin. (**b**) Gankyrin is a tumor suppressor killer. Gankyrin triggers degradation of at least six tumor suppressors by ubiquitin-proteasome system (UPS), and interacts with many important molecules, facilitating carcinogenesis

the nucleus [25], and associating with p300 to inhibit its interaction with RelA [26].

Consisting of seven ankyrin repeats, gankyrin binds many other proteins and affects many signaling pathways, contributing to carcinogenesis. Examples include MAGE-A4 [27], IGFBP-5 [28], SHP-1 [29], ATG7 [30], Keap1/Nrf2 [31], WWP2/Oct4 [32], PI3K/Akt [33], Rac1/JNK [34], β-catenin [35], Rho-A/ROCK [21], IL-6/STAT3 [36], IL-8 [37], YAP1 [38], and hypoxia-inducible factor-1α (HIF-1α) [22, 33].

Animal Models Overexpressing Gankyrin in the Liver

To assess the oncogenic activity in vivo, we produced transgenic mice that specifically overexpress gankyrin in the hepatocytes by using the hepatitis B virus X protein (HBX) promoter and serum amyloid P component (SAP) promoter [22]. Unexpectedly, both of these transgenic lines developed hepatic vascular neoplasms (hemangioma/hemangiosarcomas), but no HCCs. Further studies suggested that this was because gankyrin binds and sequester FIH-1, which results in decreased interaction between FIH-1 and HIF-1α, resulting in increased activity of HIF-1α and vascular endothelial growth factor (VEGF) production. Using the albumin promoter, Zhao et al. [34] observed HCC in the transgenic mice, but only after diethylnitrosamine (DEN) plus carbon tetrachloride (CCl_4) treatment. Recently, occurrence of spontaneous HCC was reported in transgenic zebrafish using fabp10a promoter with Tet-Off system [39].

Importance of Non-Parenchymal Cells in Carcinogenesis as Demonstrated by Gankyrin-Knockout Mice

The effects of gankyrin in the tumor microenvironment were investigated by using mice with liver parenchymal cell-specific gankyrin ablation (*Alb-Cre;gankyrin$^{f/f}$*) and gankyrin ablation both in liver parenchymal and in non-parenchymal cells (*Mx1-Cre;gankyrin$^{f/f}$*) in the DEN hepatocarcinogenesis model (Fig. 2) [40]. Gankyrin upregulated VEGF expression in tumor cells. Gankyrin bound to Src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1) which was mainly expressed in liver non-parenchymal cells, resulting in phosphorylation and activation of STAT3. Gankyrin deficiency in non-parenchymal cells, but not in parenchymal cells,

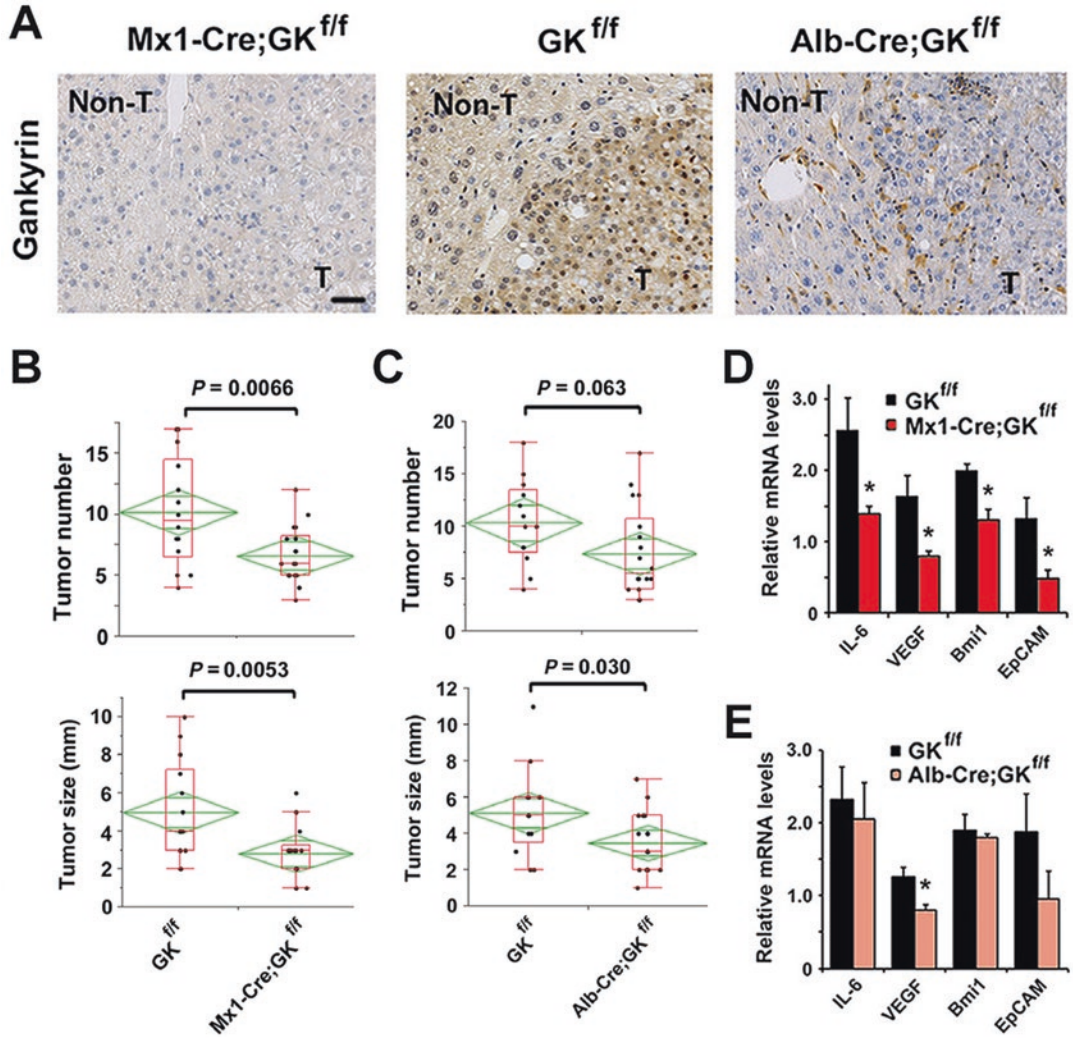

Fig. 2 Important roles in hepatocarcinogenesis played by gankyrin in non-parenchymal as well as parenchymal cells (Modified from reference [40]). (**a**) Control *gankyrin*$^{f/f}$ (*GK*$^{f/f}$), *Alb-Cre;GK*$^{f/f}$, and *Mx1-Cre;GK*$^{f/f}$ mice were challenged with DEN and killed after 8 months. Liver sections were examined with immunohistochemistry using gankyrin-specific antibody. *Non-T* non-tumorous liver tissues, *T* tumors. Scale bar, 50 μm. (**b**) Tumor number (upper) and maximal tumor sizes (diameters, lower) in *GK*$^{f/f}$ ($n = 14$) and *Mx1-Cre;GK*$^{f/f}$ ($n = 18$) mice. (**c**) Tumor number (upper) and maximal tumor sizes (diameter, lower) in *GK*$^{f/f}$ ($n = 13$) and *Alb-Cre;GK*$^{f/f}$ ($n = 18$) mice. (**d**, **e**) RNA was extracted from tumors of *Mx1-Cre;GK*$^{f/f}$ (**d**) or *Alb-Cre;GK*$^{f/f}$ (**e**) mice and *GK*$^{f/f}$ mice. Relative amounts of mRNA were determined by quantitative RT-PCR (qRT-PCR) and normalized to the amount of actin mRNA. The amount of each mRNA in the untreated liver was given an arbitrary value of 1.0. Data are means ± SEM ($n = 5$)

reduced STAT3 activity, IL-6 production, and expression of cancer stem cell markers (Bmi1 and EpCAM), leading to attenuated tumorigenic potential. These results have suggested a model as shown in Fig. 3a. Essentially similar results were obtained by analyzing mice with intestinal epithelial cell-specific gankyrin ablation (*Villin-Cre;Gankyrin*$^{f/f}$) and gankyrin ablation both in myeloid and epithelial cells (*Mx1-Cre;Gankyrin*$^{f/f}$) in the colitis-associated cancer model [29]. Significant differences were observed in tumor numbers and sizes between *Mx1-Cre;Gankyrin*$^{f/f}$

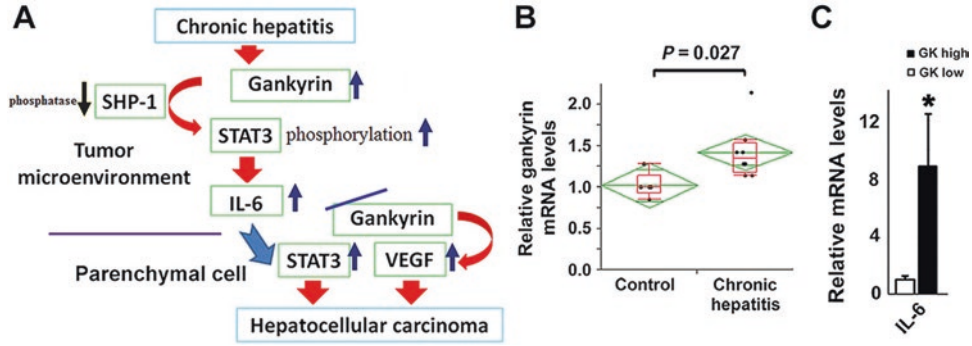

Fig. 3 Inflammation and gankyrin (Modified from [40]. (**a**) A model of the role played by gankyrin in hepatocarcinogenesis. Chronic inflammation enhances gankyrin expression in the liver. Gankyrin binding to SHP-1 leads to enhanced IL-6 production in the tumor microenvironment. The augmented inflammatory response activates STAT3, and gankyrin upregulates the expression of VEGF in tumor cells, which eventually promote the development of HCC. (**b**) Liver specimens were collected using needle biopsy in 13 patients clinically suspected of non-alcoholic steatohepatitis. The expression of gankyrin mRNA in livers without inflammation or fibrosis (control, $n = 5$) and those with inflammation and fibrosis (chronic hepatitis, $n = 8$) was determined by qRT-PCR. (**c**) Liver specimens were collected using needle biopsy before sorafenib treatment. The mRNA levels of IL-6 in HCC were determined by qRT-PCR and compared between patients grouped according to the level of gankyrin expression in hepatic non-parenchymal cells as assessed by immunohistochemistry

mice and control mice, but not between *Villin-Cre;Gankyrin*$^{f/f}$ mice and control mice. Consistent with the animal models, chronic inflammation enhanced gankyrin mRNA expression in the human liver (Fig. 3b), and protein expression in non-parenchymal cells as well as hepatocytes [40]. High gankyrin expression in non-parenchymal cells was associated with enhanced IL-6 expression in HCC (Fig. 3c), and gankyrin expression in the tumor microenvironment was negatively correlated with progression-free survival in patients undergoing treatment with Sorafenib for HCCs [40]. These findings indicate important roles played by gankyrin in non-parenchymal cells as well as parenchymal cells in the pathogenesis of liver cancers and colorectal cancers.

Gankyrin as a Promising Therapeutic Target

Gankyrin seems to be an excellent target of cancer therapy because of the following reasons:

1. Signaling interactions between cancer cells and their supporting stroma have been suggested to evolve during the course of multi-stage tumor development [41], and gankyrin promotes oncogenesis both in cancer cells and their supporting stroma [29, 40].
2. Gankyrin promotes carcinogenesis both in early (initiation, promotion) and late (progression, metastasis) stages. For example, in rat HCC model, overexpression of gankyrin starts at fibrosis stage [42], and in human liver tissues, expression is progressively increased from hepatitis, cirrhosis, adenoma to HCC [43]. In many different types of cancers including those of the liver [30], colorectum [44], esophagus [45], and lung [46], high-level expression is correlated with invasion, metastasis, poor survival, and resistance to therapy.
3. Gankyrin is overexpressed in most cases of HCC [3] and other types of cancers, including those of the esophagus [45], stomach [47], prostate [48], and colorectum [18].
4. Ubiquitous low expression of gankyrin in normal tissues, and overexpression in almost all types of cancers including those of the brain, breast, lung, ovary, prostate, and stomach.
5. Gankyrin kills multiple major tumor suppressors (Fig. 1b).

6. Gankyrin can enable the hallmarks of cancer, at least five out of six original hallmarks and two out of two additional hallmarks [41]. By inhibiting gankyrin, therefore, we can target most of the pathways supporting the hallmarks therapeutically.

Experimental Anti-Gankyrin Agents

Since gankyrin is a versatile tumor suppressor killer and its activities seem to result from the binding to various partners, inhibition of the interactions is a promising strategy for controlling cancer initiation and progression. Indeed, overexpression of MAGE-A4, a gankyrin interactor of unknown function, suppressed the tumorigenic activity of gankyrin [27]. Overexpression of C-terminal portion of S6b inhibited proliferation of malignant cells (Fig. 4a, b). Although the structure of gankyrin has been clarified, research studies focusing on structure-based drug design of gankyrin are still limited. A synthetic protein GBP7.19 [49] and a small molecular drug cjoc42 [50] have recently been reported, but direct and specific gankyrin inhibitors should be investigated further.

Another promising strategy is an inhibition of gankyrin expression. Down-regulation of gankyrin expression by small interfering RNA (siRNA) or shRNA promotes apoptosis of tumor cells in vitro [15]. Growth of human cancer cells transplanted to nude mice is suppressed by intra-tumoral injection of siRNA [18] or adenovirus delivering shRNA against gankyrin [51]. As expected, the anti-proliferative activity of shRNA against gankyrin was enhanced by simultaneous expression of p53 in p53-deficient cancer cells (Fig. 4c).

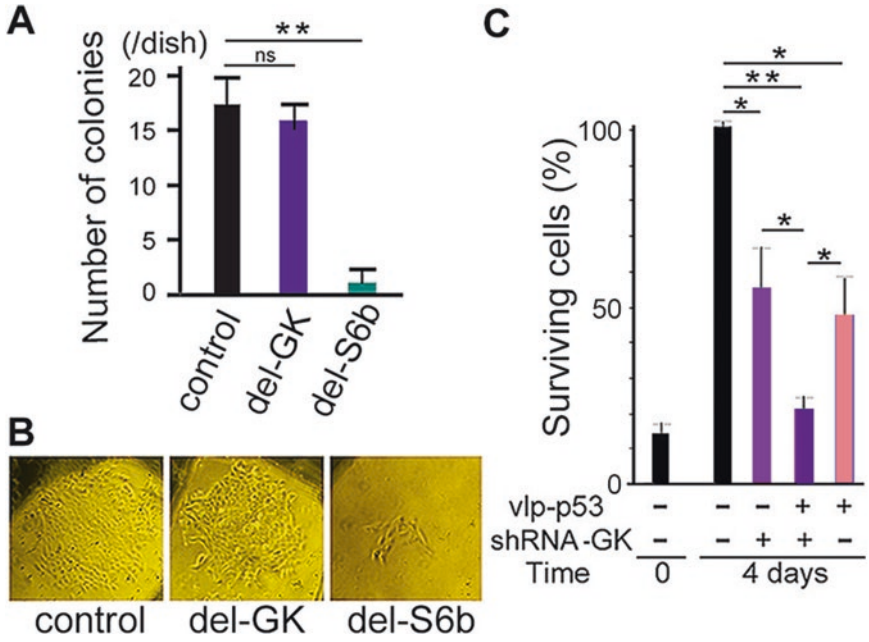

Fig. 4 Suppression of cell proliferation by gankyrin inhibitors. (**a**, **b**) Effects of overexpression of gankyrin-interacting S6b mutant. Human osteosarcoma U-2 OS cells were cultured in 6-cm dishes and transfected with plasmid DNAs (1 μg/dish) expressing mutant gankyrin with deletion of the two ankyrin-repeat motifs (del-GK), mutant S6b with N-terminus deletion (del-S6b), or control vector together with neo-resistance gene. After 8 days of culture in G418-containing medium, numbers of colonies were counted (**a**), and representative colonies were photographed under microscope (**b**). (**c**) Effects of shRNA. Human prostate cancer PC-3 cells with no wild-type p53 gene were incubated with plasmid nanoparticles expressing p53 (vlp-p53) or shRNA against gankyrin (vlp-shRNA-GK) prepared by RNTein Biotech Lab, CA. Volumes of added plasmids were adjusted with controls expressing vector alone. Four days later, surviving cell numbers were counted under microscope

Conclusion

Gankyrin plays important roles in non-parenchymal cells as well as parenchymal cells in the pathogenesis of liver cancers, colorectal cancers, and probably other cancers. Thus, by acting both on cancer cells and on the tumor microenvironment, anti-gankyrin agents are promising as therapeutic and preventive strategies against various cancers. As response rate of HCC to systemic chemotherapy is only 0 to 25%, blocking expression and/or function of gankyrin might be especially valuable in human HCCs. For the development of therapeutics, in vitro human cell transformation systems that incorporate the effects of non-parenchymal cells and gankyrin would be useful.

Gankyrin, a small ankyrin-repeat protein, has many activities with proteins controlling the cell cycle, transcription, apoptosis, and then many signaling pathways. How much of these activities are dependent on chaperoning the assembly of, and delivering the ubiquitylated substrates to the 26S proteasome, and how many are dependent on gankyrin being present in much smaller complexes with other proteins to control various cell processes, e.g., cell growth signaling pathways? The resolution of these questions must be answered to move toward a fuller understanding of gankyrin actions in the cell.

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Contributing Roles of CYP2E1 and Other Cytochrome P450 Isoforms in Alcohol-Related Tissue Injury and Carcinogenesis

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Abstract

The purpose of this review is to briefly summarize the roles of alcohol (ethanol) and related compounds in promoting cancer and inflammatory injury in many tissues. Long-term chronic heavy alcohol exposure is known to increase the chances of inflammation, oxidative DNA damage, and cancer development in many organs. The rates of alcohol-mediated organ damage and cancer risks are significantly elevated in the presence of co-morbidity factors such as poor nutrition, unhealthy diets, smoking, infection with bacteria or viruses, and exposure to pro-carcinogens. Chronic ingestion of alcohol and its metabolite acetaldehyde may initiate and/or promote the development of cancer in the liver, oral cavity, esophagus, stomach, gastrointestinal tract, pancreas, prostate, and female breast. In this chapter, we summarize the important roles of ethanol/acetaldehyde in promoting inflammatory injury and carcinogenesis in several tissues. We also review the updated roles of the ethanol-inducible cytochrome P450-2E1 (CYP2E1) and other cytochrome P450 isozymes in the metabolism of various potentially toxic substrates, and consequent toxicities, including carcinogenesis in different tissues. We also briefly describe the potential

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implications of endogenous ethanol produced by gut bacteria, as frequently observed in the experimental models and patients of nonalcoholic fatty liver disease, in promoting DNA mutation and cancer development in the liver and other tissues, including the gastrointestinal tract.

Keywords
Alcohol · Acetaldehyde · CYP2E1 · Oxidative stress · Inflammation · DNA mutation · Cancer

Introduction

Long-term chronic heavy alcohol (ethanol) intake is known to increase the incidences of cancer in many tissues, including the liver, mouth, esophagus, gastrointestinal tract, pancreas, prostate, and female breast [1–6]. The alcohol-mediated cancer rates are significantly increased in the presence of co-morbidity factors such as smoking, viral and bacterial infections, carcinogens, and potentially harmful diets, such as poor nutrition, western-style high fat diets, and soft drinks containing high fructose corn syrup. We have previously reviewed that the rates of alcohol-mediated cancer in experimental rodent models and alcoholic people are increased by the one or combinations of the following risk factors: (1) formation of etheno-(or acetaldehyde)-DNA adducts; (2) elevated production of reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxides, and metabolic conversion of procarcinogens to carcinogens via ethanol-inducible cytochrome P450-2E1 (CYP2E1); (3) accumulation of iron leading to increased ROS generation, lipid peroxidation, mutation of p53 gene or its covalent modifications of its protein; (4) decreased cellular levels of antioxidant glutathione (GSH) and S-adenosylmethionine (SAMe), resulting in oxidative stress and DNA hypomethylation of oncogenes and epigenetics changes; (5) depletion of retinoic acid with consequent cell proliferation through activation of activator protein-1 (AP-1); (6) activation of an inflammatory cascade via increased intestinal barrier dysfunction, resulting in endotoxemia, activation of tissue macrophages, including hepatic Kupffer cells via Toll-like receptor-4 (TLR-4), oxidative stress, the nuclear factor-KappaB (NF-kB) or early growth response-1 (Egr-1) activation, and production of inflammatory cytokines and chemokines; (7) reduced number and/or function of Natural Killer cells; (8) decreased activities of antioxidant enzymes and DNA repair enzymes. Since these areas have been covered in our previous review [1] and others [2–6], we specifically focus on the updated roles of CYP2E1-related oxidative stress, leaky gut, and metabolic activation of potentially toxic substrates in DNA adduct formation and cancer development in this chapter. The important roles of the ethanol-induced CYP2E1 and other cytochrome P450 isozymes in the metabolisms of various substrates and consequent cytotoxicities, including acetaldehyde and other carcinogenic substances in many tissues, are briefly summarized. Additive and/or synergistic interactions between ethanol and other risk factors toward increased levels of DNA adducts and carcinogenesis are also described. In addition, the potential roles of the endogenous ethanol and acetaldehyde produced by bacteria in certain tissues, as frequently observed in rodent models and patients with nonalcoholic fatty liver disease (NAFLD) and/or steatohepatitis (NASH), in promoting inflammatory tissue injury and cancer development are briefly discussed. Finally, based on the mechanistic study results, we have described the translational opportunities against alcohol-associated injury and carcinogenesis in many tissues.

Updated Mechanisms of Ethanol-Mediated Carcinogenesis

The International Agency for Research on Cancer (IARC) has concluded that both ethanol and its oxidative metabolite acetaldehyde are human

carcinogens [7]. One of the major factors for the increased cancer development in alcoholic individuals and ethanol-exposed animal models could be increased oxidative and nitrative stress, through activation of many pro-oxidant enzymes with decreased contents of small molecule antioxidants and suppressed antioxidant enzymes. In fact, chronic excessive alcohol intake is known to increase oxidative and nitrative stress which can be produced through impaired mitochondrial electron transport chain (i.e., mitochondrial dysfunction), elevated levels of the CYP2E1, NADPH oxidases, the inducible form of nitric oxide synthase (iNOS), xanthine oxidase, etc. [8–17]. In addition, chronic alcohol ingestion is known to decrease the levels of many small molecule antioxidants glutathione (GSH), *S*-adenosylmethionine (SAMe), folic acid, many vitamins, including retinol (vitamin A), thiamine (vitamin B1), ascorbic acid (vitamin C), vitamin D (ergocalciferol and cholecalciferol), α-tocopherol (vitamin E), menadione (vitamin K3) through insufficient absorption in the GI tract, suppression of biosynthesis, and increased metabolic degradation [1, 8, 18–21]. The depletion of these enzyme cofactors and co-enzymes can exert dramatic influences on major metabolic pathways and genetic/epigenetics changes. Moreover, the activities of antioxidant enzymes such as mitochondrial low-Km aldehyde dehydrogenase-2 (ALDH2), glutathione peroxidase (Gpx), superoxide dismutase (SOD), methionine adenosyltransferase-1 (MAT1), and catalase can be significantly suppressed through oxidative modifications in alcohol-exposed tissues [22–25]. It is likely that the decreased levels of antioxidants and suppressed antioxidant enzymes or proteins render the host more susceptible to inflammatory tissue injury and carcinogenesis [1–4].

Alcohol (ethanol) is not a strong carcinogen compared to its oxidative metabolite acetaldehyde. However, alcohol-induced carcinogenesis is significantly increased in the presence of a risk factor such as smoking, western-style high fat fast food, and viral infection. Elevated levels of highly reactive acetaldehyde and lipid aldehydes, through suppressed ALDH2 activity or genetic mutation in the *ALDH2* gene, as observed in many people in East Asian countries [26–28] or in various tissues of *Aldh2*-null mice exposed to alcohol gavages [29] can increase the amounts of DNA adducts and cancer. Alternatively, increased amounts of acetaldehyde produced from activated ADH through mutation of its gene [30] can interact with the amine groups of deoxyguanosine (dG), deoxyadenosine (dA), and deoxycytosine (dC) to generate N^2-ethylidene-DNA and more stable N^2-etheno-DNA adducts [31–33], contributing to increased mutagenesis and cancer development, as recently reviewed [34]. In the presence of reducing agents, such as polyamines, acetaldehyde dimer crotonaldehyde can interact with DNA and produce a stronger mutagenic propano-DNA adduct [35]. Furthermore, under increased oxidative stress following alcohol exposure, the amounts of lipid peroxides are significantly elevated and some of the highly reactive lipid aldehydes such as acrolein (ACR), malonaldehyde (MDA), MDA-Acetaldehyde (MDA-AA), and 4-hydroxynonenal (4-HNE) can interact with DNA, producing mutagenic DNA adducts, leading to increased carcinogenesis [36–38]. Analyses of human specimens revealed that the levels of the lipid-aldehyde DNA adducts in the liver and mucosa of the esophagus and colon in alcoholic people appear to depend on the levels of CYP2E1. In contrast, these adducts in some patients with NASH do not correlate with the CYP2E1 levels and are likely derived from inflammation-driven oxidative stress, as reviewed [39]. Furthermore, the rates of carcinogenesis could be markedly increased when p53 and DNA repair enzymes, such as oxoguanine DNA glycosylase (Ogg1), are inactivated in alcohol-exposed rodents [40, 41]. Although the molecular mechanisms for the inactivation of DNA repair enzymes in alcohol-exposed rodents have not been studied in detail, it is likely that these enzymes could be oxidatively modified and thus inactivated under increased oxidative and nitrative stress.

Contributing Roles of CYP2E1 and Other P450 Isoforms in Tissue Injury and Cancer Development

Multiple Regulations of CYP2E1 and Alcohol-Related Tissue Injury and Carcinogenesis

It is well-established that ingested ethanol is primarily metabolized by alcohol dehydrogenase (ADH, Km for ethanol 0.8–1 mM) expressed in the liver, esophagus, stomach, and intestine. However, after chronic alcohol exposure or intake of large amounts of ethanol, a significant amount of alcohol is also metabolized by another enzyme system so-called the microsomal ethanol oxidizing system (MEOS), consisting of CYP2E1, CYP1A2, and CYP3A with CYP2E1 being a major component [8, 9, 42, 43]. In fact, under higher blood alcohol concentration (BAC) up to ~100 mM, as observed in some alcoholics [44], ethanol-induced CYP2E1 (Km for ethanol 8–10 mM) becomes important in the oxidative metabolism of ethanol, producing acetaldehyde, which can impair intestinal barrier function and produce DNA adducts, contributing to inflammatory tissue injury [45] and carcinogenesis [34], respectively. Unlike other P450 enzymes, CYP2E1, a loosely bound enzyme to the ER membrane, exhibits NADPH oxidase activity, thus producing ROS during its catalytic cycle or even in the absence of its substrate, as reviewed earlier [8, 25, 42, 43]. The ROS include superoxide anion, hydrogen peroxide, and hydroxyethyl radical, depending on the local environment, including the presence of iron, which is known to be accumulated by alcohol exposure [46], and other preexisting conditions, contributing to DNA damage and carcinogenesis. In addition, CYP2E1 is known to produce RNS in certain conditions despite little induction of iNOS [47]. CYP2E1, present in both endoplasmic reticulum (ER) and mitochondria [48, 49] in the liver and extra-hepatic tissues such as kidney, colon, and brain [50], is induced and activated by acute or chronic exposure to alcohol and other small molecules such as acetone and isoniazid or pathophysiological conditions such as fasting and diabetes through different regulatory mechanisms [8, 18, 51–53]. Moreover, its level and activity are increased in obese and/or hyperglycemic diabetic rodents and in humans [8, 18, 52–55]. Because of different induction mechanisms of CYP2E1 (e.g., protein stabilization by ethanol or acetone [50, 56–58]), increased mRNA translation by isoniazid and pyridine, and mRNA increase by fasting, western-style high fat diet, over-feeding obesity, or diabetes [52–55], the overall levels and activities of CYP2E1 are expected to be increased in an additive or synergistic manner [8, 18, 59, 60]. For example, alcohol exposure in diabetic rodents and people would markedly elevate CYP2E1 activity, thus producing greater levels of oxidative stress and tissue injury [15, 18, 59]. Another example of additive or synergistic effect is interactions between alcohol drinking and other risk factors such as western-style high fat diet [52], nicotine [61], infection with hepatitis viruses [62], and certain chemicals or carcinogens, which are CYP2E1 substrates, such as dimethylnitrosamine (DMN) [56], diethylnitrosamine (DEN) [63], urethane, and benzene [64, 65], promoting acute toxicity or inflammatory tissue injury, as reviewed [15, 18, 59, 66]. Consequently, the degree of these interactions with cellular macromolecules, including DNA, is significantly increased in fasting or other pathological conditions with lower GSH levels [67], making the host more susceptible to oxidative DNA damage and mutations, contributing to inflammatory tissue injury and carcinogenesis.

In addition to the oxidative ethanol metabolism, CYP2E1 is known to metabolize many small molecule environmental toxicants and potential carcinogens, some of which are the inducers of CYP2E1 [51, 65]. The exogenous CYP2E1 substrate compounds are thioacetamide (TAA), acetaminophen (APAP), isoniazid, cisplatin, halothane, isoflurane, salicylic acid, solvents (e.g., ethylene, carbon tetrachloride, chloroform, dichloromethane, benzene, pyridine, and toluene), various long-chain fatty acids, DMN, DEN, bromodichloromethane, Vitamin A derivatives (retinoic acid), and others [51, 63–65]. Endogenous substrates of CYP2E1 can be acetone, long-chain

fatty acids, glycerol, 4-HNE, and others, including ethanol and acetaldehyde produced by oral or gut bacteria [18, 68–72]. Metabolism of these substrates by CYP2E1 and consequent organ damage appear to positively correlate with the levels of CYP2E1 activity, with a few exceptions of APAP- or carbon tetrachloride (CCL4)-exposed models, as reviewed [14, 18, 24, 66]. For instance, clinically relevant doses of APAP, halothane, thioacetamide, or CCL4 can cause acute drug-induced liver injury (DILI) or toxicity via alcohol and drug interactions, especially in alcohol-exposed or fasted individuals or rodents with increased CYP2E1. The APAP- or CCL4-mediated hepatic (and/or kidney) injury is initiated through their metabolism by CYP2E1, since pretreatment with CYP2E1 inhibitors or *Cyp2e1*-null mice was fully protected from these types of DILI or acute toxicity [24, 47, 73]. The decreased levels of retinoic acid by CYP2E1-mediated metabolism [74] and substrate competition with ethanol may also contribute to elevated hepatocyte proliferation and liver tumor progression in alcohol-exposed rodents and alcoholic individuals [74, 75]. By using knockout mice deficient of a specific pro-oxidant enzyme, Bradford and colleagues demonstrated that CYP2E1 but not NADPH oxidase is important in promoting alcohol-mediated DNA damage [76]. In this model, the levels of etheno-DNA adduct were significantly decreased in ethanol-exposed *Cyp2e1*-null mice compared to those of the wild-type mice. In contrast, the elevated levels of ethanol-related DNA adducts were unchanged and still observed in the corresponding *NADPH-oxidase*-null mice. In addition, the levels of exocyclic ethanol-DNA adduct were significantly increased in CYP2E1-overexpressing HepG2 cells upon ethanol exposure [77] and some patients with alcoholic fatty liver and fibrosis [78]. The levels of these DNA adducts can be significantly decreased in the presence of chlormethiazole (CMZ), a specific CYP2E1 inhibitor [75, 77]. Furthermore, the elevated levels of exocyclic ethanol-DNA adduct observed in experimental rodents were also observed in the biopsied esophagus specimens of human alcoholic patients with esophagus cancer [79]. The levels of etheno-DNA adduct significantly correlated with cell proliferation, which was markedly increased in people who both drank and smoked [80]. All these results strongly indicate an important role of CYP2E1 in producing carcinogenic etheno-DNA lesions in the experimental model and alcoholic individuals [75–77, 81].

Chronic inflammation plays an important role in cancer development and progression of malignant states [82–84]. A recent long-term epidemiological study with more than 121,000 health professional men and women revealed that consumption of pro-inflammatory diet is associated with colon cancer, underscoring the important role of inflammation in carcinogenesis [85]. Cancer-associated inflammation and inflammation-derived DNA lesions and malignancies seem to be genetically stable [36] and can be affected by the extrinsic and intrinsic factors. For instance, extrinsic factors, such as alcohol intake, smoking, viral and bacterial infections, exposure to environmental toxicants and pro-carcinogens, and pathophysiological conditions, can increase inflammation and cancer risk. Additionally, cancer-causing mutations can stimulate inflammatory reactions by activating and recruiting inflammatory cells through various pro-inflammatory cytokines and chemokines or complementary factors [82–84]. Additionally, it is well-established that excessive chronic alcohol intake can cause chronic inflammation through increased intestinal barrier dysfunction and endotoxemia [86–89]. Elevated plasma levels of bacterial endotoxin lipopolysaccharide (LPS) can interact with TLR-4 in the Kupffer cells, leading to inflammatory liver injury and carcinogenesis [82, 90]. It is possible that plasma LPS, a potent inducer of iNOS and nitration of many cellular proteins [89, 91, 92], can further stimulate inflammation and injury to the GI tract and other tissues. Recent data suggest that binge alcohol can stimulate gut leakiness and inflammatory liver injury in a CYP2E1-dependent manner [89, 93, 94]. The elevated CYP2E1 was responsible for increased oxidative and nitrative stress, causing nitration of several intestinal tight and adherent junction proteins [93]. Nitrated junctional complex proteins were degraded by

proteolytic degradation following ubiquitin conjugation. The markedly decreased amounts of the intestinal junctional complex proteins in binge ethanol-exposed rats compared to control counterparts were confirmed by quantitative mass-spectral analysis [93]. Subsequently, the levels of the gut junctional complex proteins were significantly decreased and contributed to leaky gut and endotoxemia in ethanol-exposed rodents and people who died suddenly due to heavy alcohol intoxication compared to people who died from nonalcoholic causes [93]. These events of ethanol-mediated leaky gut and inflammatory liver injury were not observed in the corresponding *Cyp2e1*-null mice or were significantly attenuated in the ethanol-exposed wild-type mice co-treated with CMZ, a specific inhibitor of CYP2E1 [93] or an antioxidant *N*-acetylcysteine (NAC) [89]. Consequently, elevated levels of serum LPS can upregulate TLR4 in the liver, stimulating inflammation and hepatic injury, including fibrosis, potentially leading to carcinogenesis [82, 95, 96]. Furthermore, both extrinsic and intrinsic inflammation are known to modulate or suppress immune responses, which can provide a suitable environment for alcohol-induced carcinogenesis and tumor progression, as reviewed [83, 84].

Distribution of CYP2E1 and Carcinogenesis in Extra-Hepatic Tissues

The majority of CYP2E1 is expressed in the liver. However, it is also expressed in many extrahepatic tissues such as kidney [50, 97], brain [50, 98, 99], lymphocytes [100], lung [101, 102], pancreas [103], nasal mucosa [104], esophagus [105], stomach [105], intestine [50, 93, 94], and female breast [106]. Induction of CYP2E1 (and other P450 isozymes) following exposure to ethanol, potentially environmental toxicants, or under pathophysiological conditions such as fasting and diabetes, is likely to result in production of ROS and RNS which can lead to increased levels of DNA adducts, inflammatory tissue injury, and carcinogenesis [75–81, 93, 94]. Furthermore, CYP2E1 was shown to stimulate post-translational modifications followed by inactivation of various proteins in different subcellular organelles, resulting in ER stress, mitochondrial dysfunction, and inflammatory cell death of many tissues, as reviewed [14, 18, 66, 92]. In many cases, the levels of tissue injury, DNA adducts, or cancer positively correlated with those of CYP2E1 [30–32, 61, 73–79, 93, 94, 107–109]. In contrast, no or little correlation between the severity of tissue injury and the CYP2E1 level was observed in some other cases [95, 97, 110]. However, the lack of correlation between the levels of CYP2E1 and DNA adduct or tissue injury does not necessarily rule out the important role of CYP2E1 because of its permissive role to allow other proteins or genes to exhibit their damaging effects, as described in some experimental models [95, 97, 110, 111].

Contribution of Other P450 Isoforms in Alcohol-Related Tissue Injury and Carcinogenesis

Most of the studies on alcohol-related DNA mutations and carcinogenesis appear to focus on the correlative roles of ADH, CYP2E1, and ALDH2 involved in the oxidative metabolism of ethanol and acetaldehyde [75–81, 96, 112]. However, it has been demonstrated that chronic ethanol exposure can induce CYP2E1 as well as other cytochrome P-450 isoforms, such as CYP1A1 [104], CYP2A5 [111, 113], and CYP3A [114, 115]. The levels of these P450 isoforms induced by ethanol exposure may be small compared to those of CYP2E1 induction. However, we also need to pay attention to their contributing roles in promoting DNA mutation, tissue injury, and caner in both liver and extra-hepatic tissues such as esophagus, gastrointestinal tract, nasal cavity, and lung in alcoholic individuals and/or alcohol-exposed rodents especially in the presence of another co-risk factors such as tobacco smoking and potentially harmful drugs, toxicants, or solvents. For instance, CYP1A1 is known to metabolize carcinogens aryl hydrocarbons [AHs] or polyaromatic hydrocarbons (PAHs) [116], such as benzo[a]pyrene and 3-methyl-cholanthrene, contained in charred foods and cigarette tars or smokes. Consequently,

ethanol-mediated induction of CYP1A would lead to increased levels of DNA adducts and carcinogenesis in the liver and other extra-hepatic tissues such as nose, esophagus, and lung. Similarly, ethanol-mediated inductions of CYP2A5 can increase DNA adducts in the lung since it is known to metabolize a tobacco carcinogen, the tobacco-related nitrosamine-related carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [117]. Likewise, alcohol-mediated induction of CYP3A4 [114, 115] and CYP2A5 [118] may render the host with increased levels of DNA adducts and carcinogenesis caused by a mycotoxin aflatoxin B1 (AFB1) [119]. In addition, elevated CYP3A isozymes are likely to metabolize many drugs, including tamoxifen, leading to their activation and production of DNA adducts and carcinogenesis in certain endometrial tissues such as uterus [120]. Furthermore, elevated CYP3A and other P450 isoforms may accelerate metabolic clearance of many drugs, including anti-retroviral agents, leading to oxidative stress and cellular injury [121, 122] in HIV-1-infected people, who exhibit higher rates of hepatic cirrhosis and cancer [123]. In fact, alcohol-mediated elevation/activation of other P450 isoforms in the esophagus, GI tract, nasal cavity, and lung is likely involved in increased DNA mutation, inflammatory injury, and cancer in these tissues in the presence of another risk factor, like smoking, and/or exposure to other potentially toxic substances, such as benzene and toluene [63–65], or western-style high fat fast foods [95].

Increased DNA Adducts, Inflammatory Tissue Injury, and Carcinogenesis in NAFLD/NASH Through the Production of Endogenous Ethanol and Acetaldehyde

In the previous section, we have focused on DNA mutations, inflammatory tissue injury, and carcinogenesis in alcoholic individuals and alcohol-exposed rodents. However, it is now known that people with NAFLD/NASH are more susceptible to DNA damage and cancer in the liver [124] and many extra-hepatic tissues, including the GI tract [125]. Some main reasons for increased DNA damage and cancer could be overgrowth of gut bacteria, increased alteration of gut microflora (dysbiosis), mucosal inflammation, oxidative/nitrative stress, and leaky gut after exposure to western-style high fat fast foods, fructose-rich soft drinks, and metabolic syndromes [124–128]. In addition, it was shown that ethanol and acetaldehyde can be endogenously produced in obese rodents with NAFLD [70, 129] and some people, including children with NAFLD/NASH [71, 129–131] without exogenous ethanol intake. Moreover, production of acetaldehyde was demonstrated in various bacteria present in the mouth [68, 69, 132], lung [132, 133], and GI tract, including colon [70, 129, 134]. Consequently, the levels of etheno-DNA adduct, inflammatory injury, and carcinogenesis could be increased in these tissues in rodents and people with NAFLD/NASH, as reviewed [124, 125]. For instance, gut dysbiosis with the increased population of the ethanol-producing bacterial family *Enterobacteriaceae*, including *Escherichia coli*, can lead to increased production of ethanol, local inflammation, and leaky gut, contributing to endotoxemia and inflammatory tissue injury, as demonstrated with pediatric patients with NASH [129, 131]. Although not studied, it would be of interest to know whether gut CYP2E1 is induced by the endogenously produced ethanol, albeit small amounts compared to those of alcohol intake. If gut CYP2E1 is induced in people or rodents with NAFLD/NASH, it may cause oxidative stress and oxidatively modify the intestinal junctional complex proteins, resulting in decreased amounts of the intestinal junctional complex proteins in alcohol-exposed rats and mice, as recently demonstrated [93, 94]. These events caused by the endogenous ethanol in NAFLD/NASH [135] may contribute to leaky gut, endotoxemia, and inflammatory tissue injury accompanied with DNA damage. Although the mechanisms for increased DNA mutation and carcinogenesis remain to be further studied, it is likely that CYP2E1 and other P450 isoforms, which can be induced by the endogenously produced ethanol, may be involved in the oxidative metabolisms of ethanol and acetaldehyde. In addition, these P450 isoforms can metabolize

pro-inflammatory substances n-6 long-chain fatty acids and/or other environmental toxicants or potential pro-carcinogens such as PAHs contained in charred western-style fast foods, contributing to elevated DNA adducts and carcinogenesis in the GI tract and other organs. Based on the damaging roles of the endogenously produced ethanol and acetaldehyde with potentially elevated CYP2E1 and other P450 isoforms, it is expected that people with diabetes or NAFLD/NASH could be more susceptible to DNA damage, inflammatory tissue injury, and cancer especially when they drink even small amounts of alcohol through additive or synergistic interactions [136, 137].

Translational Research Opportunities

Alcohol and acetaldehyde are human carcinogens [7]. The incidences of alcohol-related inflammatory tissue injury, DNA mutation, and carcinogenesis are significantly increased in the presence of another risk factor(s). As mentioned earlier, these risk factors are smoking, viral and bacterial infections, pro-inflammatory western-style high fat fast foods with fructose-containing soft drinks, poor nutrition, and preexisting pathophysiological conditions such as fasting and diabetes [18, 59, 66, 92]. Simultaneous exposure to these risk factors is likely to decrease cellular antioxidants, such as GSH and SAMe, and inactivate many antioxidant enzymes. Consequently, the rates of oxidative stress, lipid peroxidation, gut leakiness, endotoxemia, inflammatory tissue injury, DNA damage, and carcinogenesis would be increased [8, 18, 66, 92]. Based on these mechanisms, prevention or moderation of alcohol drinking would be the best remedy for alcohol-related tissue injury and carcinogenesis. Unfortunately, it would be difficult to decrease alcohol intake in many addicted alcoholic individuals. If alcohol drinking is not prevented, we may consider using adequately balanced diets with antioxidants (such as NAC) [89] or dietary supplements such as n-3 docosahexaenoic acid (DHA) [23], garlic compounds, including diallyl sulfide [37], resveratrol [138], walnut [139], indole-3-carbinol [140], ellagic acid [141], and pomegranate [94], many of which were shown to reduce or suppress the amount or activity of CYP2E1. As reported earlier [89, 93, 94, 140, 142], decreased CYP2E1 would lead to prevention of oxidative stress, leaky gut, and inflammatory tissue injury. Administration of soy protein isolate was also shown to protect from alcohol-mediated tumor promotion in DEN-exposed mice [143]. In addition, eubiosis by administering probiotics *Lactobacillus* [144] and *Bifidobacterium* strains [145] may be considered. In fact, a recent study showed that supplementation with *Akkermansia muciniphila* prevented alcohol-mediated intestinal barrier dysfunction and inflammatory liver injury through the gut–liver axis [146]. Furthermore, treatment with synthetic chemical inhibitors of CYP2E1, such as CMZ [75, 77, 89] and YH439 [147], can be considered to mitigate alcohol- and acetaldehyde-mediated inflammatory tissue injury, DNA mutation, and carcinogenesis.

Conclusion

As reviewed previously, both chronic and acute alcohol intake can change many different metabolic pathways and immunological dysregulations along with genetic and epigenetic changes. In the liver, alcohol drinking stimulates fatty liver, inflammation, fibrosis, cirrhosis, and cancer [148]. The development and progression of chronic liver disease usually depend on the amounts and duration of alcohol intake as well as the presence of another co-morbidity risk factor(s). Alcohol or acetaldehyde-mediated cancer in extra-hepatic tissues may also depend on the amounts of DNA adducts of the pro-carcinogens by CYP2E1 and other P450 isoforms-mediated metabolisms that can be increased by exposure to alcohol and/or another environmental toxicant(s). In this chapter, we have briefly summarized the biochemical properties of CYP2E1 and its roles in ethanol and acetaldehyde metabolism. We have also described its multiple regulations, tissue distribution, and causal roles in alcohol-mediated gut leakiness, inflammation, apoptosis, tissue injury, DNA

mutation, and carcinogenesis. In addition, we have mentioned the potential roles of other P450 isoforms, which are also induced or activated by alcohol or another environmental toxicant, in metabolizing potentially harmful substances, contributing to increased carcinogenesis in the liver and extra-hepatic tissues. The causal roles of CYP2E1 and other P450 isoforms in stimulating inflammatory tissue injury, DNA adducts, and carcinogenesis would be significantly increased in the presence of another risk factor such as smoking and/or western-style high fat fast foods. We have also briefly described the newly emerging roles of the gut microbiome changes and the endogenously produced ethanol in promoting DNA adduct formation and disease progression despite the absence of exogenous alcohol intake. Based on the understanding of the mechanisms of increased carcinogenesis, we have described potential methods of preclinical translational opportunities by preventing alcohol drinking or using dietary supplements, including various naturally occurring antioxidants and probiotics, and chemical inhibitors of CYP2E1. In fact, many drug candidates are being evaluated for preventing or treating liver disease through targeting the gut–liver axis [144–146, 149, 150]. Based on the important roles of gut microbiome changes in promoting leaky gut, endotoxemia, inflammatory tissue injury, some of these drug candidates may become a good candidate as an anti-cancer agent.

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Part II

Prostate Cancer Progression

Novel Human Prostate Epithelial Cell Cultures

Johng S. Rhim

Abstract

Prostate cancer is the most common male cancer in the USA and the second leading cause of male cancer death in the USA. African American men have higher incidence and mortality rate from prostate cancer compared to Caucasian men in North America, indicating the prostate cancer is a major public health problem in this population. Studies of prostate cancer have been hampered by various factors including (1) restricted access to tissues, (2) difficulties in propagating premalignant lesions and primary prostate tumors in vitro, and (3) limited availability of prostate cell lines for in vitro studies. There is no commercially available pair of non-malignant and tumor cells derived from the same prostate cancer patient. Primary prostate epithelial cells grow for a finite life span and then senesce. Immortalization is defined by continuous growth of otherwise senescing cells and is believed to represent an early stage in tumor progression. To examine these early stages, we have developed in vitro models of prostate epithelial cell immortalization. Generation of human primary epithelial (HPE) cells has been achieved using the serum-free keratinocyte growth medium. Retrovirus containing human telomerase reverse transcriptase (hTERT) was also used for the generation of primary non-malignant and malignant tumor cells. In addition, we have established the first immortalized cell lines of a pair of non-malignant and malignant tumors derived from an African American prostate cancer patient. Interestingly, we have found that the Rock inhibitor and feeder cells induced the conditioned reprogramming (CR) of epithelial cells—normal and tumor epithelial cells from many tissues to proliferate indefinitely in vitro, without transduction of viral or cellular genes. More recently, using CR, we have established normal and tumor cultures respectively from a patient prostatectomy. These CR cells grow indefinitely in vitro and retain stable karyology. The tumor-derived CR cells produced tumors in SCID mice. The use of novel pair of non-malignant and malignant tumor cells derived from the same patient provides a unique in vitro model for studies of early prostate cancer and for testing preventive and therapeutic regimens.

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Keywords

Prostate cancer · In vitro models · hTERT · HPV-16 E6E7 · Rock inhibitor · Feeder cells

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Generation of Short-Term Cultures Derived from Benign and Prostate Cancer Patients by Use of the Keratinocyte Serum-Free Growth Medium

As described previously [1], our Prostate Cancer Cell Center successfully generated more than 100 primary prostate epithelial cells from tumors of prostate cancer patients as well as normal prostate tissue of the same patients using our established protocol. Keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Gaithersburg, MD) was used for growing and maintaining the cells. We found that it is very useful in growing and maintaining primary HPE cells and for short-term cultures of primary HPE cells (Table 1). The early passage growing cells before senesce were cryopreserved. The cryopreserved cells were found to be very useful for further studies.

Establishment and Characterization of Non-malignant and Tumor-Derived Primary Prostate Epithelial Cells Immortalized by Telomerase

Despite Barrows et al. [2] attempts, which spanned for more than a century, only a few cell lines derived from human prostate cancer have been produced. To date, only three human prostate cancer cell lines (DU-145, PC-3, and LNCaP) are commercially available and well-studied. All were derived from metastatic lesions and thus have a void in reagents representing primary localized adenocarcinoma of the prostate, and it is unlikely that they accurately reflect the genetic makeup or biological behavior of primary prostate tumor. However, their use has greatly contributed to our understanding of human prostate carcinogenesis and progression. Better understanding of the use of recombinant DNA technology and telomerase resulted in the establishment of novel primary benign and malignant tumor-derived HPE cell lines during the past decade. We have successfully been able to establish and characterize novel five immortalized HPE cell lines derived from benign prostate tissues as well as prostate cancer tissues using telomerase, the gene that prevents senescence, for the first time [3]. Furthermore, we have succeeded in the establishment of HPE cell model for the study of prostate cancer in high-risk population, one on African American prostate cancer and the other on familial prostate cancer.

Telomerase is an enzyme responsible for replicating telomere and is composed of an RNA subunit containing an integral catalytic subunit, human telomerase reverse transcriptase (hTERT). It has been well implicated that telomerase is the escape from cellular senescence. Transfection of hTERT into human cell type can itself induce immortalization. Interestingly, telomerase expression in human cells does not induce change associated with a transformed phenotype or an altered genetic phenotype. The phenotypic characterization of hTERT immortalized primary non-malignant and malignant tumor-derived HPE cell lines has been studied. Expression of malignant phenotype was examined for cell lines by evaluating their proliferation rates and their ability to grow above the agar layer, to form cell aggregates and colonies in soft agar and in vivo in SCID mice and their sensitivity to androgen. The results obtained have demonstrated that these hTERT-immortalized HPE cell lines retained the original phenotypes of the primary cells and express some of their prostate-specific markers. The androgen-responsive properties of some cell lines should help answer questions related to androgen regulation of prostate cells. These novel in vitro models may offer the study of prostate carcinogenesis and also provide the

Table 1 Primary cell strains generated at Prostate Cell Center (2007)

Number of cell strains generated from prostate tumors of patients	142
Number of cell strains generated from benign and tumors from the same patients	110
Number of cell strains generated from African American patients	22
Number of cell strains generated from familial prostate cancer patients	48

means for testing both chemoprevention and chemotherapeutic agents. We have also identified the putative prostate stem cell markers CD133 and CXCR4 in these hTERT-immortalized primary and malignant tumor-derived HPE cell lines [4]. Furthermore, we have shown that telomerase-immortalized non-malignant HPE cells retained the properties of multipotent stem cells. However, our further attempts to immortalized HPE cell with hTERT were disappointed since its success rate was very low. The reason is not clear and has to be investigated.

Establishment of a Pair of Non-malignant and Malignant Tumor-Derived Cell Lines from an African American Prostate Cancer Patient

African American men have a 60% higher incidence, more aggressive and more mortality rate from prostate cancer compared to Caucasian men in North America [5]. We need to study largely and urgently to better understand why African American men develop more aggressive forms of the disease and why they are more likely to die from it. The etiology of these racial differences in clinical manifestation of prostate cancer is unclear; hormonal, genetic, behavioral, and environmental factors have all been implicated [6]. To understand the many factors suspected of contributing to the development of this malignancy, there is a critical need for in vitro model representing primary tumors. However, no suitable in vitro models which accurately reflect the in situ characteristics of malignant epithelium for the study of African American prostate cancer are available.

To date, only three reports have demonstrated the establishment of African American prostate cancer-derived cell lines; MDA PCa 2a and MDA PCa 2b cell lines were derived from a single bone metastasis in 1997 [7]. These cell lines exhibit androgen-independent growth in vitro and in vivo; however, they retain androgen responsiveness. A second cell line was derived from primary localized adenocarcinoma of the prostate. The E006AA cell line was established as spontaneously immortalized cells from a patient with a clinically localized prostate cancer. However, this cell line shows androgen-dependent growth but is not tumorigenic in nude mice in 2004 [8]. In 2014 the establishment and characterization of a highly tumorigenic subline of the previously described non-tumorigenic E006AA cell line [8] have been shown [9]. Here we succeeded for the first time the establishment of a novel pair of non-malignant and malignant tumor derived from an African American prostate cancer patient [10].

We introduced a retrovirus construct expressing HPV-16 E6E7 into third-passaged RC-77/E and RC-77/T cells through overnight infection. Non-infected cells could not be propagated serially beyond five subcultures. In contrast, the HPV-16 E6E7-infected RC-77N/E and RC-77T/E cells have an apparently unlimited lifespan and have been successfully subcultured for more than 40 passages over the course of 1 year with no evidence of decreased proliferation capacity (Table 2). The RC-77N/E and RC-77T/E cells had typical epithelial morphology and are growing in the keratinocyte serum-free growth medium. The RC-77T/E cells form three dimensional spheroids, whereas the RC-77N/E cells do not form the spheroids. The RC-77T/E cells produced tumors in SCID mice, whereas the RC-77N/E cells produced no tumors in SCID mice. Both cells expressed NKX 3.1, CK8, AR, and p16 but did not express PSA in mRNA level underlying monolayer culture (Table 2). They

Table 2 Properties of RC-77T/E and RC77N/E cell lines

	RC-77T/E	RC-77N/E
Life span (>40 passages)	>40	>40
Gene expression by RT-PCR		
E6	+	+
NKX3.1	+	+
Cytokeratin 8	+	+
AR	+	+
P16	+	+
PSA	+	–
GAPDH	+	+
3D organoid formation	+	–
Tumorigenicity in SCID mice	3/3	0/3

Table 3 Karyological characterization of RC-77T/E and RC-77N/E cell lines

Cell line (Passage)	RC-77T/E (P-39)	RC-77N/E (P-39)
Ploidy	Near diploid human male	Near diploid human male
Chromosome count	45–48	45–48
Modal number	46	47
Normal chromosomes	Single X and Y in each karyotype	Single X and Y in each karyotype
Marker chromosomes	8	6
	M1 = del (4)(q28q34) + hsr in some	M1 = del(4)(q28q34) + hsr in some
	M1A = t(4q;?)	M1A = t(4q;?)
	M1B = del/t(4?)(q28;?)	M2 = der(9?)
	M2 = der(9?)	M2A = del(M2p−)
	M3 = iso(22?)	M3 = iso(22?)
	M4 = der(22?)	M4 = der(22?)
	M5 = 16q+	
	M6 = 15p+	

showed androgen responsiveness when treated with synthetic androgen (R-1881).

Both cell lines are cytogenetically similar near diploid human male (XY). However, RC77T/E cell line has new marker chromosomes (M5 = 16q+ and M6 = 15p+) in addition to those observed non-tumorigenic RC-77N/E cell line (M1 = del(4)(q28q34) + hsr in some, M1A = t(4q:?), M2 = der(9?), M2A = del(M2p−), M3 = iso(22?), and M4 = der (22?)) (Table 3). Some of the alteration of chromosomes observed in RC-77T/E and RC-77N/E cell lines have been already reported in literature. The presence of a marker chromosome involving in chromosome 4 (M1 = del(4)(q28q34) + hsr in some) in both cell lines has been reported in other African American-derived metastatic prostate cancer cell line (MDA PCa) and a primary African American prostate cancer cell line, E006AA [7, 8]. However, the observation of a common karyotypic pattern (regional deletion of 13q.5q.16q and 8p and gain of 8q and 5q) of primary prostate cancer derived from African American men and Caucasian American men led to the conclusion that biological interracial difference among the prostate cancer patients is not due to gross chromosomal alteration [11]. The determination of specific genetic markers involved in African American prostate cancer needs to be further studied.

It is interesting to note that 4q alteration observed in both cell lines also observed chromosome changes in an established human prostate cell line derived from primary tumor of a familial prostate cancer patient [12]. Possible evidence of a prostate cancer susceptibility loss on chromosome 4q has been reported [13]. As has been described, new marker chromosomes (M5 = 16q+ and M6 = 15p+) were observed in tumorigenic RC-77T/E cell line in addition to those observed in non-tumorigenic RC-77N/E cell line (M1 = del(4)(q28q34) + hsr in some, M1A = t(4q;?), M2 = der(9?), M2A = del(M2p−), M3 = iso(22?), and M4 = der(22?)) (Table 3). Interestingly, the marker chromosome 15+ has been observed in a primary tumor-derived human prostate cancer cell line [14], whereas the marker chromosome 16q is the most frequent region alteration observed in a primary prostate cancer [15, 16].

The results obtained have been demonstrated that these immortalized non-malignant and primary prostate tumors of the same patient-derived cell line retained their original phenotypes and express some of their prostate-specific markers. Additionally, these models retained their androgen-responsive properties which ultimately will be useful to answer questions of therapeutic target at AR. Furthermore, this is the first African American primary prostate cancer cell line that retains the ability to generate tumors in vivo. In addition, this is the first pair of non-malignant and tumor cells derived from the same African American prostate cancer patient. These novel findings fill much needed void in prostate cancer and health disparity research. To our knowledge, this is the only model with such properties and may offer unique opportunity for the study of early stage of African American prostate cancer development.

ROCK Inhibitor and Feeder Cells Induce the Conditional Reprogramming (CR) of Epithelial Cells

In the 1950s George Gey succeeded in a method to grow cells from cervical cancer patient tumor, Henrietta Lacks (HeLa). It became an important laboratory model for cancer research and in understanding tumor biology for drug development. It would go on to contribute significant advances in scientific research, leading to two Nobel Prizes and aid in the development of vaccines, cancer treatments, in vitro fertilization, and a genome sequence that was published last year in 2017. The cells have been used in the research of toxins, hormones, and viruses and to study the effects of radiation and the development of the polio vaccine. Approximately 70,000 peer-reviewed papers have been published using them. There are 17,000 US patents that involve HeLa cells established from a metastatic tumor. To date, no commercial normal and cancer paired cell lines derived from the same patient are available. Most primary cell cultures regardless of the numerous methods used to sustain them suffer from limited lifespan due to lack of understanding of requirements for long-term stem cell maintenance and the inability to recapitulate the essential stem cell niche in vitro. These factors lead to gradually decrease in proliferation rate and senescence. One of the biggest challenges in current cancer biology research is the development of a method to generate stable HPE cell lines from primary normal epithelium and tumors.

Several approaches have been used to overcome cellular senescence in primary cell culture for nearly seven decades:

In 1970 it has been shown that irradiated or non-dividing mitomycin-treated mouse fibroblast cells as a feeder layer lead the support to 30–50 passages rather than 5–6 passages [17].

The most common method of immortalizing is using viral genes (SV40 and HPV-16 or 18) [18–20]. SV40 large T antigen was used for many cell types in 1994, but leads to genomic instability with results after a few passages. The cultured transformed cells lose the properties of the cells from which were derived.

Telomerase immortalization of normal human somatic cells by ectopic overexpression hTERT [21] has been shown in 1998. hTERT does not lead to s tumorigenic transformation of normal cells [3, 14], thus becoming the method of choice for immortalizing primary cell culture. However, the success rate is low as we described in primary HPE cells.

In 2009, an additional approach was the use of Rho kinase (ROCK) inhibitors. This has been to keep embryonic and somatic cells growing in culture and to maintain induced pluripotent stem (iPS) cells in undifferentiated cells [22]. None of these approaches have produced stable permanent cell lines.

In 2010, we have found that Rock inhibitor (a Rho kinase inhibitor Y-27632) and fibroblast feeder cells induced the conditional reprogramming (CR) of epithelial cells [23]. The CR cells allow the establishment of patient-derived normal and tumor epithelial cells from many tissue types including breast, lung, colon, and prostate without transduction of exogenous viral or cellular genes [23] (Fig. 1, Table 4).

We demonstrated that a Rho kinase inhibitor (Y-276320), in combination with fibroblast feeder cells, induces normal and tumor epithelial cells from many tissues to proliferate indefinitely in vitro, without transduction of exogenous viral and cellular genes. Primary prostate and mammary cells, for example, are reprogrammed toward a basaloid, stem-like phenotype [24] and form well-organized prostaspheres and mammospheres in Matrigel [25]. However, in contrast to the selection of rare stem-like cells, the described growth conditions can generate 2×10^6 cells in 5–6 days from needle biopsies, and can generate cultures from cryopreserved tissue and from fewer than four viable cells. Continued cell proliferation is dependent on both feeder cells and Y-27632, and the conditionally reprogrammed cells (CRCs) retain a normal karyology and remain non-tumorigenic. This technique also efficiently establishes cell cultures from human and rodent tumors. For example, CRCs established from human prostate adenocarcinoma

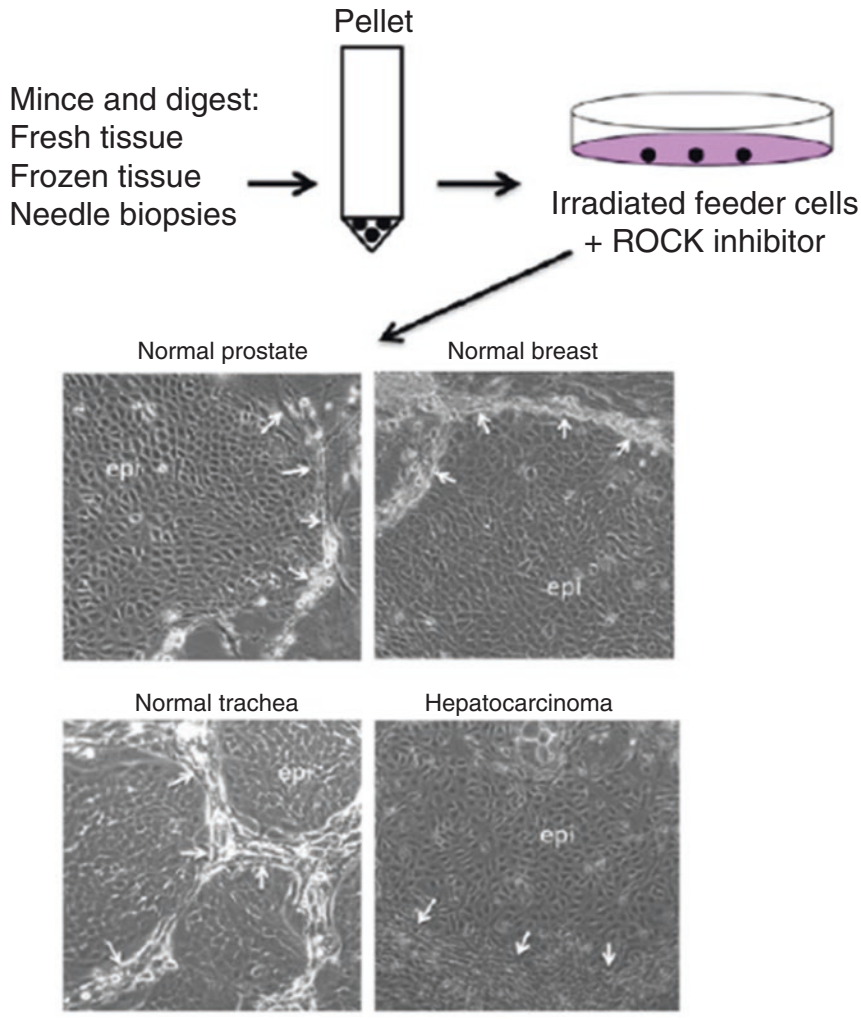

Fig. 1 Propagation and immortalization of human adult epithelial cells. Prostate, breast, tracheal, and liver (hepatocellular carcinoma) tissues were harvested and digested with trypsin-collagenase, Cells isolated from the tissues were plated on a feeder layer of irradiated (3000 rad) Swiss 3T3 cells (J2 subclone) and grown in F medium containing 10 μmol/L ROCK inhibitor (Y-27632). Small colonies could be observed after 1 day. At day 5 (shown), there were large islands of epithelial (epl) cells that compressed the surrounding feeder cells (*white arrows*)

Table 4 CR cells established from primary human prostate cells

(1) Like hTERT immortalization, this method does not transform normal cells and maintains normal karyotypes for 33 passages from normal prostate cells and maintains a normal phenotype
(2) Reprogrammed to ward a basaloid stem-like phenotypes
(3) Form well-organized prostaspheres and mammospheres in Matrigel
(4) Needle biopsy generates 2×10^6 cells in 5–6 days
(5) Generate culture from cryopreserved tissue
(6) Retain a normal karyotype
(7) Non-tumorigenic
CR cells established from human prostate adenocarcinoma
(1) Display the instability of chromosome 13
(2) Proliferate abnormality in Matrigel
(3) Form tumors in SCID mice

displayed in stability of chromosome 13, proliferated abnormally in Matrigel, and formed tumors in mice with severe combined immunodeficiency. The ability to rapidly generate many tumor cells from small biopsy specimens and frozen tissue provides significant opportunities for cell-based diagnostics and therapeutics and greatly expands the value of biobanking. In addition, the CRC method allows for the genetic manipulation of epithelial cells ex vivo and their subsequent evaluation in vivo in the same host.

The mechanism for this reprogramming is unclear, although there are insight parallels with the process of cell immortalization induced by the HPVs. The high-risk HPVs encode two oncoproteins, E6 and E7, that are required for the efficient immortalization of primary cells. One of the most critical roles for the E6 protein in cell immortalization is the induction of hTERT [23, 26, 27].

We have shown that the predominant factor for hTERT induction in CRCs is grown in F medium with feeder cells and that Y-27632 has a minimal contribution to the induction. The mechanism by which telomerase is induced by the F medium plus feeder cells is to be investigated. The second component of HPV-mediated cell immortalization is the function of the E7 protein which inactivates the Rb pathway. E7 has recently remodeled the actin cytoskeleton [28] and inactivated Rho [29, 30]. We believe that the combination of F medium containing feeder cells and Y-27632 supplies two distinct functions that promote cell proliferation: (1) Induction of telomerase and (2) cytoskeletal remodeling and/or interference with the p16/Rb pathway (Fig. 2).

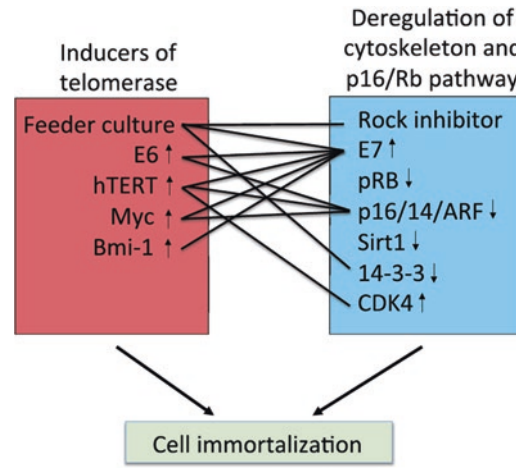

Fig. 2 Potential model for the cooperative effects of F medium plus feeder and the ROCK inhibitor in cell immortalization inducers of telomerase and deregulation of cytoskeleton and p16/Rb pathway. Class 1 genes induced telomerase activity and class 2 genes alter both the p16/Rb pathway and the cell cytoskeleton. The connecting lines indicate documented interactions between class 1 and class 2 genes that result in cell immortalization

Conditionally Reprogrammed (CR) Normal and Primary Tumor Prostate Epithelial Cells: A Novel Same Patient-Derived Cell Model for Studies of Human Prostate Cancer

As our previous study demonstrated, CR cells allow the establishment of patient-derived normal and tumor epithelial cell cultures from a variety of tissue types including breast, lung, colon, and prostate [23].

Using CR cells we have recently succeeded in establishing matched normal (GUMC-29) and tumor (GUMC-30) cultures from the same prostate cancer patient, respectively [31]. These CR cells proliferate indefinitely in culture and retain stable karyotypes. More importantly, only tumor-derived GUMC-30 cells produced tumors in SCID mice, demonstrating maintenance of the critical tumor phenotype. Characterization of cells with DNA fingerprinting demonstrated identical patterns in normal and tumor CR cells as well as in SCID mice xenografted tumors. By flow cytometry, both normal and tumor CR cells expressed basal, luminal, and stem cell markers, with the majority of the normal and tumor CR cells expressing prostate basal cell markers, CD44 and Trop2, as well as luminal marker, CD13, suggesting a transit-amplifying phenotype. Consistent with this phenotype, real-time RT-PCR analyses demonstrated that CR cells predominantly expressed high levels of basal cell markers (KRT5, KRT14, and p63) and low levels of luminal markers. When the CR tumor cells were injected into SCID mice, the expression of luminal markers (AR, NKX3.1) increased significantly, while basal cell markers dramatically decreased. These data suggested that CR cells maintain high

Table 5 Properties of GUMC-29 and GUMC-30 cell lines

CR normal culture (GUMC-29) and tumor culture (GUMC-30) were established from the same prostate cancer patient
The CR cells grow indefinitely and retain stable karyology
GUMC-30 cells only produce tumors in SCID mice
DNA finger printing: Identical patterns in normal CR cells, tumor CR cells, and in xenografted tumors
Flow cytometry: Both normal and tumor cells
Positive in basal, luminal, and stem cell markers with the majority of both CR cells
Positive prostate basal cell markers, CD44 and Trop2, and also luminal marker CD13, suggesting a transit-amplifying phenotype
RT-PCR analysis: Predominantly high level of basal cell markers (KRTS, KRT14, and p63) and lower levels of luminal markers
The CR tumor cells injected into SCID mice: The expression of luminal markers (AR, NKX3.1) increased significantly while basal cell markers dramatically decreased
This data suggested that CR cells maintain high level of proliferation and low level of differentiation in the presence of feeder cells and ROCK inhibitor but undergo differentiation once injected into SCID mice
Genomic analyses (SNP and INDEL) identified gene matched in tumor cells include component of apoptosis, cell attachment, and hypoxia phenotype

levels of proliferation and low levels of differentiation in the presence of feeder cells and ROCK inhibitor, but undergo differentiation once injected into SCID mice. Genomic analyses, including SNP and INDEL, identified genes mutated in tumor cells, included components of apoptosis, cell attachment, and hypoxia pathway (Table 5). The use of novel matched patient-derived cells provides a unique in vitro model for the studies of early prostate cancer.

Conclusion

As we have described, the use of keratinocyte serum-free medium is a very efficient method for the generation of primary HPE cells. We have generated five immortalized HPE cell cultures derived from both benign and malignant tissues of prostate cancer patient with telomerase. Examination of these cell lines for their morphologies, proliferative capacities, response to androgen stimulation, growth above the agar, and formation of tumors in SCID mice suggests that they may serve as valid and useful tools for the elucidation of early events in prostate tumorigenesis. In addition, we have identified putative stem cell markers CD133 and CXCR4 in these hTERT-immortalized primary non-malignant and malignant tumor-derived HPE cell lines. We have shown the first documented case of the establishment of a pair of non-malignant and malignant tumor derived from an African American prostate cancer patient with HPV-16 E6 E7 genes. The model will provide a novel tool to study the molecular and genetic mechanisms of prostate carcinogenesis especially for high-risk African American men. ROCK inhibitor (Y-27632) and fibroblast feeder cells induce the conditional reprogramming of epithelial cells from many tissues to proliferate indefinitely. In addition, using CR, we have established matched normal and tumor cultures derived from the same patient prostatectomy specimen. The matched patient-derived cells will provide a unique novel in vitro model for studies of cancer biology, discovery of biomarker, anti-cancer drug, and cancer precision medicine.

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African-American Prostate Normal and Cancer Cells for Health Disparities Research

Nicole Nicolas, Geeta Upadhyay, Alfredo Velena, Bhaskar Kallakury, Johng S. Rhim, Anatoly Dritschilo, and Mira Jung

Abstract

Prostate cancer is the most frequently diagnosed solid malignancy in men. Epidemiological studies have shown African-American men to be at higher risk for developing prostate cancer and experience higher death as compared to other ethnic groups. Establishment of prostate cancer cell lines paired with normal cells derived from the same patient is a fundamental breakthrough in cell culture technology and provides a resource to improve our understanding of cancer development and pertinent molecular events. Previous studies have demonstrated that conditional reprogramming (CR) allows the establishment and propagation of patient-derived normal and tumor epithelial cell cultures from a variety of tissue types. Here, we report a new AA prostate cell model, paired normal and cancer epithelial cells from the same patient. "Tumor" cell culture AA-103A was derived from malignant prostate tissues, and "normal" cell culture AA-103B was derived from non-malignant prostate tissues from the prostatectomy specimen of an African-American male. These paired cell cultures have been propagated under CRC conditions to permit direct comparison of the molecular and genetic profiles of the normal epithelium and adenocarcinoma cells for comparison of biomarkers, enabling patient-specific pathological analysis, and molecular and cellular characterization. STR confirmed human origin albeit no karyotypic abnormalities in the two cell lines. Further quantitative PCR analyses demonstrated characteristic markers, including the high level of basal cell marker, the keratin 5 (KRT5) in normal cells and of luminal marker, the androgen receptor (AR) as well as the programmed death-ligand 1 (PD-L1) in tumor cells. Although 3-D sphere formation was observed, the AA-103A of tumor cells did not generate tumors in vivo. We report these paired primary epithelial cultures under CRC growth as a potentially useful tool for studies to understand molecular mechanisms underlying health disparities in prostate cancer.

Keywords

American African · Prostate cancer · Primary epithelial culture · Conditional reprogramming · Cell culture

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Prostate cancer is the most frequently diagnosed solid malignancy in American men with an estimated 164,690 new cases and 29,430 deaths in USA for 2018. According to National Center for Health Statistics, the average annual prostate cancer incidence rate was 208.7 cases per 100,000 black men, 70% higher than the rate in white men. Health disparities studies have shown a higher risk of developing prostate cancer as well as higher cancer-specific death rates in African-American (AA) men as compared to Caucasian-American (CA) men [1]. Although these observations have been attributed to differences in socioeconomic status, such as limitations in access to health care services and delay of cancer diagnosis, environmental exposures and differences in genetics also have been advanced as potential causative factors [1]. AA men were reported to show a higher incidence rate, higher prostate cancer-related mortality rate, and shorter disease-free interval following treatment for localized disease [2–4].

Characteristics of AA Prostate Cancer Cells

Studies performed at centers offering equal access for patients and adjusting for socioeconomic and lifestyle factors have reported differences in survival attributable to tumor biology [5, 6]. In a study of 35 clinical trials, 1843 prostate cancer patients demonstrated survival differences by race [5]. AA men have been reported to demonstrate higher testosterone levels than CA men [6]. Such variations in molecular signaling offer further support for the possibility that genetically based differences in the biology of AA and CA prostate cancers may underlie the observed health disparities.

The normal prostate gland epithelium contains three primary differentiated cell types: luminal, basal, and neuroendocrine cells [7]. The majority of prostate cancers are pathologically classified as adenocarcinoma and display a luminal phenotype. Studies have shown that luminal columnar epithelial cells express secretory proteins (PSA) and other markers (KRT8, KRT18, NKX3.1, and AR). Basal cells localized beneath the luminal layer express markers (KRT5, KRT14, and TP63), but express low levels of androgen receptor (AR), which binds to testosterone and regulates gene expression in normal prostate tissue and prostate cancers. A comparative analysis of malignant and benign prostate tissues from radical prostatectomy specimens has shown higher expression of AR protein in prostate cancer and benign prostate tissues in AA men than in CA men [7]. Such variations in molecular signaling offer further support for the possibility that genetically based differences in the biology of AA and CA prostate cancers may underlie the observed health disparities.

Previous studies have identified differences in gene expression in tumor biopsy specimens from AA men as compared to CA men [7–9]. Microarray analyses of 69 clinically matched prostate cancer patients demonstrated differences in gene expression profiles of prostate tumors from AA and CA men, particularly in genes affecting tumor aggressiveness and metastases [8, 9]. These observations suggest that primary epithelial cultures may offer a useful tool for discovering molecular mechanisms underlying health disparities in prostate cancer.

Establishment of a Pair of Primary Normal and Cancer Epithelial Cell Cultures from AA Prostate Tumors

To investigate a feasibility of establishing paired primary normal and cancer cell lines from prostate specimens, cells were established from radical prostatectomy specimens. The presence of prostatic adenocarcinoma was determined by an experienced pathologist on gross inspection, dissecting tissue separately for the purpose of generating a cell culture as previously described, and the presence of cancer was confirmed by light microscopy [8]. However, conventional cell culture with keratinocyte serum-free medium (K-SFM) is limited by the small number of passages that can be achieved. Expansion of cells derived from prostate tissues that retain lineage commitment and normal growth and differentiation potential has been limited [8].

The Use of Conditional Reprogramming Cellular Technology for Establishment of Paired Cancer and Normal Epithelial Cell Lines from AA Prostate Tumors

The recently developed approach with to indefinitely extend the life span of primary human keratinocytes using both mouse fibroblast feeder cells (J2) and the Rho-associated kinase (ROCK) inhibitor, Y-27632, has been extended to prostate epithelial cells [10–12]. With this conditional reprogramming (CR) technology, we have been able to establish a new AA prostate cell model paired with normal and cancer epithelial cells from the same patient, AA-103A (derived from malignant prostate tissues and referred to as "tumor") and AA-103B (derived from non-malignant prostate tissues and referred to as "normal").

Prostatectomy specimens of the AA patient were annotated with clinical data and de-identified and established as malignant (AA-103A) and normal prostate epithelial cell lines (AA-103B) under an IRB approved protocol. Cells were expanded under CRC conditions as previously reported [11]. Growth characteristics of the prostate epithelial cells are shown in Fig. 1a (2D culture) and b (3D-culture). By day 3 after plating on irradiated feeder cells, epithelial colonies had already formed, expanded, and compressed the adjacent feeder cells. The growth rate and morphological characteristics of the normal and tumor cells were similar.

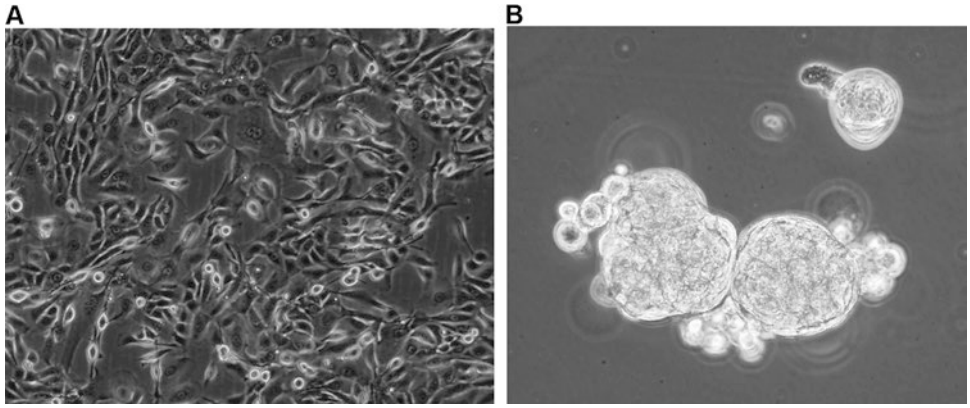

Fig. 1 Characterization of AA-103B (non-malignant—"normal") and AA-103A (cancer) cell lines. The tissues from an African-American patient (44 years old) with prostate cancer were obtained from radical prostatectomy specimens according to Walter Reed Medical Center and Uniformed Services University of the Health Sciences Internal Review Board approved protocols. Fresh prostatectomy tissue specimens were obtained under sterile conditions by an experienced pathologist and used for generating primary cell cultures as previously described [10–12]. Briefly, minced pieces of tissues were distributed to several collagen-coated cell culture dishes with keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Gaithersburg, MD). (**a**) The in vitro two-dimensional culture. The prostate epithelial cells were passaged repetitively using trypsinization techniques. The cell numbers were recorded at each passage, and a plot of population doublings versus time (days) was constructed for each cell line. Cells were grown under various conditions: CRC including both feeder cells and Y-27632 in F medium. Only cells grown in F medium containing feeders and Y-27632 continued to proliferate with a constant growth rate. (**b**) Three-dimensional (3-D) cultures. Prostate cells isolated from harvested non-malignant and cancerous tissues, respectively, were plated on a feeder layer of irradiated (4000 rad) Swiss 3 T3 cells (J2 subclone) and grown in low attached plates with F medium containing 10 μmol/L ROCK inhibitor (Y-27632). Small colonies were observed after 2 days. At day 6 there were large islands of epithelial cells that compressed the surrounding feeder cells

Identification of Cell Origin and Cytogenetic Levels

Cells grown in CRC undergo routine mycoplasma testing. To authenticate the novelty of established cell lines, we performed DNA fingerprinting. STR fingerprinting analysis, verifying that these two prostate cell cultures were derived from the same patient and were unique (Fig. 2). The prostate cell cultures have 15 identical *STR* loci and the Y-specific Amelogenin locus, thereby verifying genetic identity. DNA fingerprinting analysis at nine STR loci and at the Y-specific Amelogenin locus (Cell ID System; Promega) also showed that immortalized CRC cultures were not contaminated with another cell line during prolonged passaging. Karyotype analysis was also performed at early and late passages. The data revealed that both normal and tumor cells retained a diploid karyotype. The results for prostate cells are shown in Fig. 3 and verify that their chromosomes are structurally and numerically normal, with a 46, XY karyotype.

Molecular Characterization of AA Cell Lines

Previous studies have shown that the growth rates of the normal and tumor cells were similar; however, normal and tumor cells differed in the expression of several markers [12]. Analysis of cells taken from CRC conditions revealed that tumor cells differentiated into a luminal phenotype. To characterize both prostate normal and cancer epithelial cells, using RT-PCR we examined the expression level of marker genes associated with growth, migration, invasion, and metastases, including PD-L1, PAI1, htert, p63, KRT5, TIMP3, and AR (Figs. 4 and 5). The data showed that normal cells expressed high levels of basal markers TP63 and KRT5, but very low levels of luminal marker AR (Fig. 5). These data suggest that both phenotypes, basal and luminal, are present in CR cultures. AR, PAI1, TIM3, and PD-L1 were upregulated and p63 and KRT5 were downregulated in AA prostate cancer cells (103A). Htert

103A (p8)

DNA Analysis

	D3S1358	D7S820	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D16S539
SMP1	15, 16	10, 12	16	22	15	29, 32.2	15, 17	9, 13	11, 13	8, 9
REF	NA*	NA	NA	NA	NA	NA	NA	NA	NA	NA

DNA Analysis

	TH01	TPOX	CSF1PO	AMEL	Penta D	Penta E
SMP1	7, 9.3	8, 11	11	X, Y	7, 8	5, 11
REF	NA	NA	NA	NA	NA	NA

103B (p8)

DNA Analysis

	D3S1358	D7S820	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D16S539
SMP1	15, 16	10, 12	16	22	15	29, 32.2	15, 17	9, 13	11, 13	8, 9
REF	NA*	NA	NA	NA	NA	NA	NA	NA	NA	NA

DNA Analysis

	TH01	TPOX	CSF1PO	AMEL	Penta D	Penta E
SMP1	7, 9.3	8, 11	11	X, Y	7, 8	5, 11
REF	NA	NA	NA	NA	NA	NA

Fig. 2 Short tandem repeat (STR) analysis. Both the AA-103A and AA-103B cultures were examined for STR patterns. DNA fingerprinting of early passage (p8) prostate cells showed nine identical *STR* loci and the Y-specific Amelogenin locus, thereby verifying their genetic identity. Data are presented as mean SEM. Briefly, the analysis was performed using a commercially available kit (Cell ID System; Promega Corporation, Madison, WI), and the data were matched with cell lines in the ATCC database. The *STR* markers include *CSF1PO*, *TPOX*, *TH01*, *vWA*, *D21S11*, *D16S539*, *D7S820*, *D13S317*, and *D5S818, in addition to* the Amelogenin locus. The PCR amplification was performed according to the manufacturer's recommended protocol. Detection of the amplified fragments was achieved with the ABI 3100 genetic analyzer (Applied Biosystems). Data analysis and allele size determination were performed using GeneMapper Software (Applied Biosystems)

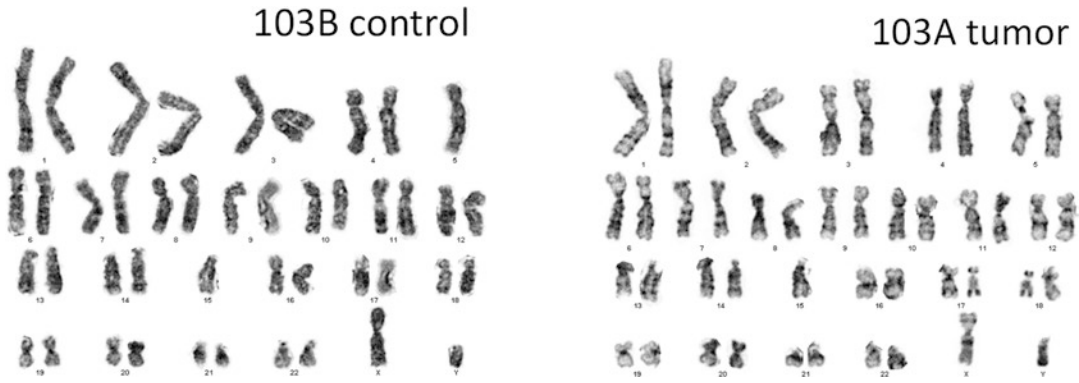

Fig. 3 Cytogenetic analysis. Chromosome counts, ploidy distribution, and Giemsa (G)-banded karyotypes were prepared by standard protocol as described previously [12]. Chromosomal analysis of normal prostate cells AA-103B revealed a normal 46, XY karyotype

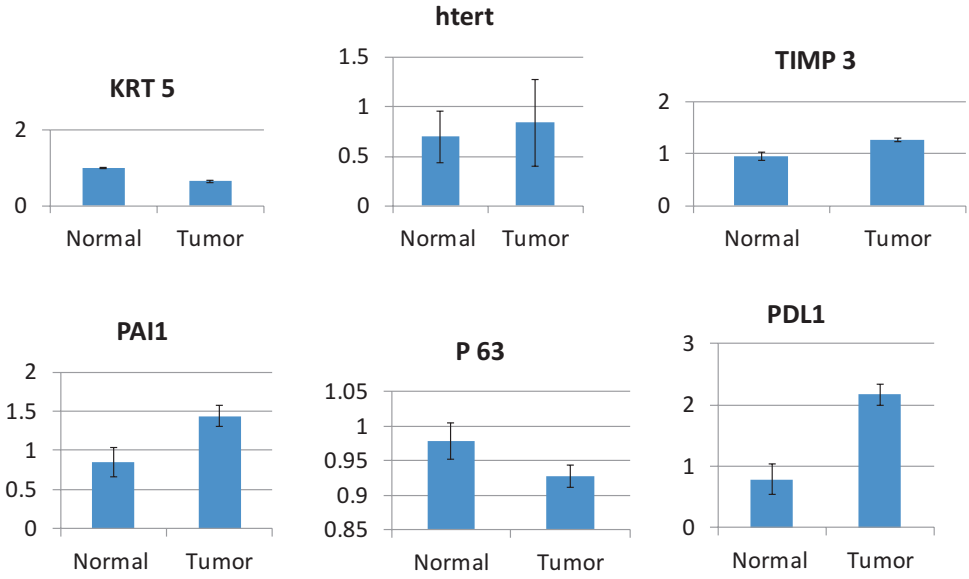

Fig. 4 Quantitative RT-PCR. Total RNA extracted from cells was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRTPCR) was performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems) on the Applied Biosystems 7900HT Fast Real-time PCR System using standard mode. Genes include AR, PAI1, KRT5, TIM3, p63, PD-L1, and Hert. GAPDH was used as an endogenous control to standardize the amount of sample added to the reaction for relative values of the amount of target cDNA

activity was similar in both cell lines. Interestingly, PAI1 and PD-L1 levels are significantly higher in tumor cells compared to normal. Furthermore, western analysis of both CR lines expressed high levels of PSA but showed a low level of AR in normal cells and higher level in tumor cells comparable to the established cancer cell line LNCaP (Fig. 5). These observations show that primary epithelial cultures may offer useful markers for discovering molecular mechanisms underlying health disparities in prostate cancer.

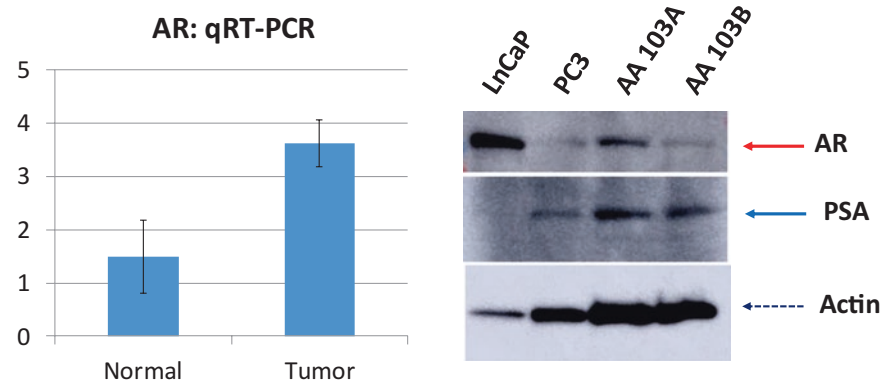

Fig. 5 The expression levels of AR gene (RT-qPCR) and protein (Western blotting). RT-qPCR was performed as described in Fig. 4. PSA and AR protein expression levels were determined by Western analysis. Actin was used as a loading control

Examination of Tumorigenicity In Vivo

To examine capacity of cells to form xenograft tumors, the prostate cancer cells were cultured in conditional medium on low attachment dishes. Within a few days, irregular spheres were formed in conditioned medium on low attachment dishes (Fig. 6a, b). Cells were then injected s.c. into nude mice. After 2 weeks, two of the five mice develop tumors at injected sites measuring 30 mm^3 and 50 mm^3. The tumors were small and after another week started to regress in size. We attributed this effect to immune responses to these cells at the injected sites in nude mice and sacrificed the mice to harvest tumors. Small tumors were obtained, and histopathologic examination did not confirm evidence of malignant growth (Fig. 6c). The experiment was repeated using severe combined immune-deficient (SCID) mice, but did not see tumor growth. We are aware that the yield of tumorigenic cancer-derived cells using CRC is in the 40% range overall.

Discussion and Conclusion

The most aggressive cancers may present as locally advanced disease precluding prostatectomy for their standard of care. However, the lack of availability of primary epithelial cultures has decelerated our understanding of molecular mechanisms underlying prostate cancer progression. Therefore, establishment of cell lines from patients with more aggressive disease is imperative.

Growing primary cultures of human prostatic epithelial cells from prostatectomy specimens is a major challenge due to the complex heterogeneity of primary tumors, a low success rate (1–10%) and technical hurdles. The slow growth rate of non-malignant epithelial cells may bias the selection of cells toward more rapidly growing phenotypes. Recently, an innovative technology, CRC, has been developed for growing primary human keratinocytes from patient's specimen using both mouse fibroblast feeder cells (J2) and the Rho-associated kinase (ROCK) inhibitor [10–12]. Appling a CRC approach, we were able to establish a new AA prostate cell model paired with normal and cancer epithelial cells from the same patient. The direct comparison of the molecular and genetic profile of the normal epithelium and adenocarcinoma cells verified their genetic identity as a human origin. Karyotype analyses revealed that both normal and tumor cells retained a diploid karyotype. Molecular characterization demonstrated that normal CR cells expressed high levels of basal cell markers, including KRT5 and TP63, but low AR. Tumor CR cells expressed a significantly high level of luminal marker, AR, as well as immune checkpoint and EMT markers (PD-L1,

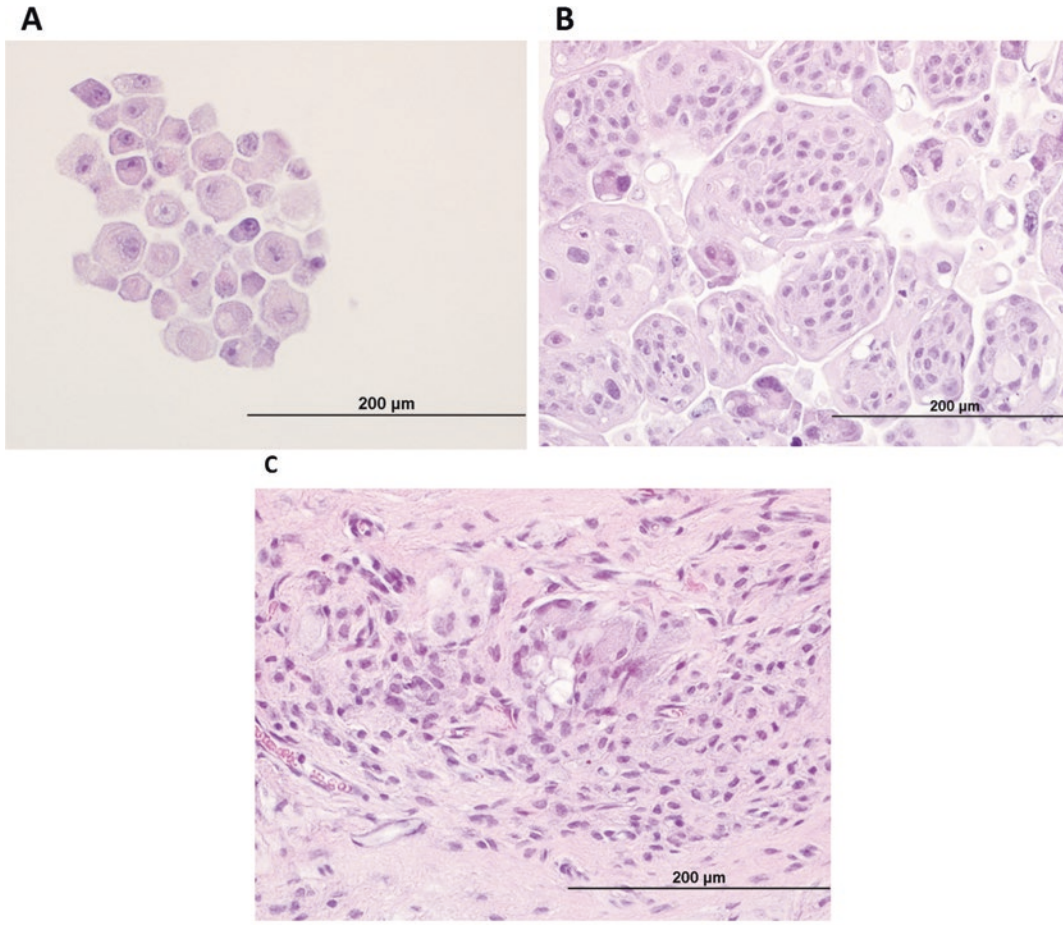

Fig. 6 Tumorigenicity in SCID mice. (**a**) Prostate CRCs in 2D culture in FY medium, 40X; these cells were used to make spheres. (**b**) Prostate CRCs in sphere condition, 40X. The spheres were cultured in conditioned medium without Y-compound on low attachment dishes. The spheres were injected s.c. into nude mice. After 2 months, two of the five mice develop tumors at injected sites measuring 30 mm^3 and 50 mm^3. (**c**) Histopathologic examination of tumors. The spheres were injected in nude mice. The prostate cancer cells made irregular spheres. Small tumors were subjected to histopathologic examination but did not confirm evidence of malignant growth

TIMP3, and PAI1), while basal cell markers are dramatically decreased. However, tumors were not generated in vivo albeit luminal markers were expressed, consistent with findings that the yield of tumorigenic cancer-derived cells is in the 40% range overall.

Tissues derived from AA patients provided the initial reagents for proof-of-principle studies establishing and characterizing paired AA cell lines. The cells may be useful for enhancing research capabilities of basic scientists and/or provide novel tools to pharmaceutical companies for preclinical oncology studies and drug development relevant to health disparities. Overall, the studies performed with cells provide proof of principal for developing additional prostate cell lines for research to facilitate the reduction of cancer health disparities.

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Assessing the Advantages, Limitations and Potential of Human Primary Prostate Epithelial Cells as a Pre-clinical Model for Prostate Cancer Research

Fiona M. Frame, Amanda R. Noble, Peter O'Toole, Jo Marrison, Timothy Godden, Andrew O'Brien, and Norman J. Maitland

Abstract

Choosing an appropriate cell model(s) is the first decision to be made before starting a new project or programme of study. Here, we address the rationale that can be behind this decision and we summarize the current cell models that are used to study prostate cancer. Researchers face the challenge of choosing a model that recapitulates the complexity and heterogeneity of prostate cancer. The use of primary prostate epithelial cells cultured from patient tissue is discussed, and the necessity for close clinical-academic collaboration in order to do this is highlighted. Finally, a novel quantitative phase imaging technique is described, along with the potential for cell characterization to not only include gene expression and protein markers but also morphological features, cell behaviour and kinetic activity.

Keywords

Prostate cancer · Cellular heterogeneity · Cell models · Quantitative phase imaging · Patient tissue

Introduction

The foundation of all studies, whether to test novel therapeutic agents or to dissect molecular signalling pathways, is the cellular model that we choose (Fig. 1). Ultimately, every model has its advantages and limitations but all too often perhaps a model is chosen because of cost, convenience and accessibility first, with biological relevance coming lower down the list. Despite several drugs showing promise after pre-clinical testing, many clinical trials fail [1, 2], and this is after testing in cell models as well as in xenografts [3, 4]. Thus, this would argue that there is a need for more effective pre-clinical models to give greater chance of success, which would in turn mean improved patient benefit and reduction of wasted funds. Over the years several researchers have met the challenge to generate better and more relevant cellular models for prostate cancer [5–8]. However, with prostate cancer, as with most cancers, a single model cannot be used to answer all questions.

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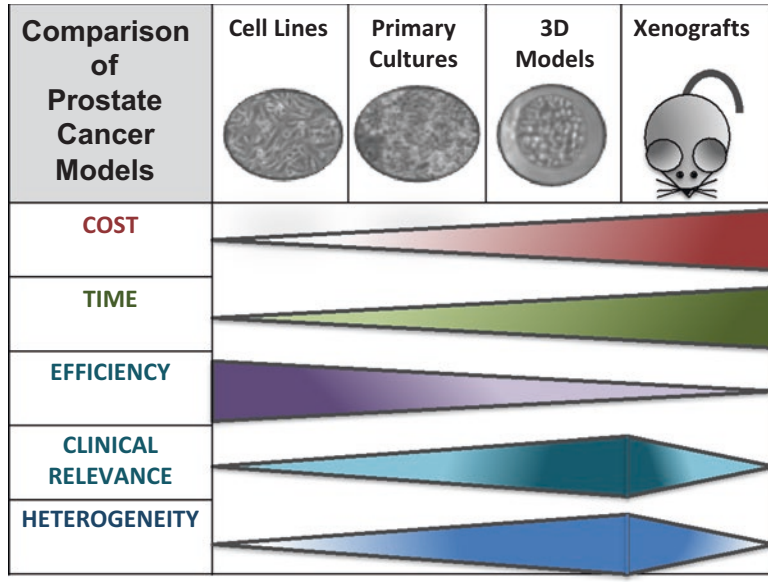

Fig. 1 Prostate cancer models: every model has advantages and limitations

Current Models for Prostate Cancer Research

Prostate cancer research has relied heavily on a few cell lines. A quick Pubmed search shows >8000 references using LNCaP cells, ~4000–5000 for PC3 and DU145 cells and several others (22RV1, RWPE-1, VCaP) coming in at a few hundred references or fewer. There is a perception in the field that we need more cell lines for prostate cancer, but the concern is that new cell lines may not get disseminated globally and picked up by the prostate community and therefore their potential is not realized; this may already be the case for existing ones. Cell lines can be divided into four main groups, cells that are—(1) immortalized by viral oncogenes (e.g., HPV and SV40) [5, 9–11], (2) cultured from xenograft tumours [5, 9, 10], (3) derived from metastatic lesions (e.g., ascites, lymph nodes and bone) [5, 9, 10] and (4) immortalized using hTERT [12–15]. The latter is an important group because the cell phenotype is retained and there are no oncogenic changes associated with this method of immortalization [16]. Also, this method has produced pairs of cell lines (normal and cancer), which are useful for comparison studies [12, 14].

The popularity of LNCaPs is obvious since for decades the design of new therapeutics has been focused on improving androgen deprivation drugs. However, it has been known from the beginning that androgen deprivation was never going to be the whole answer to prostate cancer treatment [17]. Castration-resistant prostate cancer and metastatic prostate cancer remain stubborn foes. More recently, with the advent of ever more sophisticated cell separation and genomic techniques, there have been leaps in understanding of the complexity of prostate cancer and its evolutionary progression [18–24].

Addressing Prostate Cancer Heterogeneity

The challenge with prostate cancer is to have cell line models that represent normal prostate, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), localized cancers of varying Gleason grades, aggressive and invasive cancers from within the prostate, metastatic cancers, hormone-responsive cancers, castration-resistant cancers and neuroendocrine cancers. And importantly there is also a need to have cell lines representing different races due to the

higher incidence of prostate cancer in African American men [25]. In addition to the different disease states, severity and locations, there is further heterogeneity to take into account. Prostate cancer is a multifocal disease, so there may be more than one tumour in each patient [26] and there is inter-patient variability. Along with this there is cellular heterogeneity within each tumour, multiple gene mutations, gene fusions and epigenetic changes [27]. Finding a cellular model(s) to address all of these parameters is challenging. However, understanding and acknowledging heterogeneity is critical in terms of addressing diagnosis, treatment, resistance and recurrence [28–30].

Using Primary Prostate Epithelial Cells as a Cell Model

Primary cell cultures derived from human prostate tumours have the potential to be excellent cellular models to study the disease. They are clinically relevant, representative of current disease and are not difficult to grow [31–37]. However, one limitation is that they can usually only be obtained from the prostate and not metastatic sites. Using primary cells also opens up the possibility of testing multiple patients thereby taking into account patient variation. However, this also leads to the challenge of assessing and interpreting the variation in response that is observed. How do we decide how many patient samples is enough within a single study? In addition, it is advisable to only use them at low passages, which limits the number of assays that can be done on each sample.

A controversy with use of primary prostate patient cells is the potential for normal cells to overgrow cancer cells [38, 39]. However, several studies have observed differences between normal and cancer primary prostate epithelial cells such as differential expression of matrix metalloproteinases, integrins, E-cadherin and behaviour in collagen I gels [32, 40–42]. When retrieving our samples, we have a dedicated tissue procurement officer who samples the prostate post-radical prostatectomy based on diagnostic MRI scans and trans-rectal ultrasound (TRUS)-guided biopsies, taking a needle core from a palpable tumour. Our own studies have shown that cancer cultures are more invasive than benign cultures [43], are positive for the TMPRSS2:ERG fusion at low passages [44] and samples from high Gleason grades respond differently to drug than benign and low Gleason grade samples [45].

One major issue within the field is the lack of standardization in terms of culture media between laboratories. There are differences in terms of the supplements used, some being chosen for their ability to boost cell growth [32, 33] and others to maintain a stem/progenitor population within the culture [31, 43, 46]. This makes comparisons between studies more challenging.

Undertaking a continual assessment and more complete characterization of primary epithelial cells in culture is needed. The advent of new techniques means that it is both possible and necessary to re-visit the characterization of these models. For this reason we are currently collecting both transit amplifying (progenitor) and committed basal cells (more differentiated) cultured from six samples each of Normal, BPH, Gleason 6, Gleason 7(3 + 4), Gleason 7(4 + 3), Gleason 8(4 + 4) and Gleason 9 tissues for RNA sequencing. This should hopefully identify new markers related to disease status.

Using patient cells for a variety of future studies will ultimately determine the utility of these cell cultures and provide support for strengthening clinical-laboratory relations as a research strategy. However, if more labs are to access primary patient material there has to be the will, the funds and the continuity to make a network of relationships work (Fig. 2).

Using Quantitative Phase Imaging (QPI) to Characterize a Cell Model and Address Heterogeneity

Cell characterization usually relates to gene expression patterns, protein markers and cell behaviour such as invasive potential. However, in order to also take into account cell heterogeneity when considering the effect of a drug or radiation

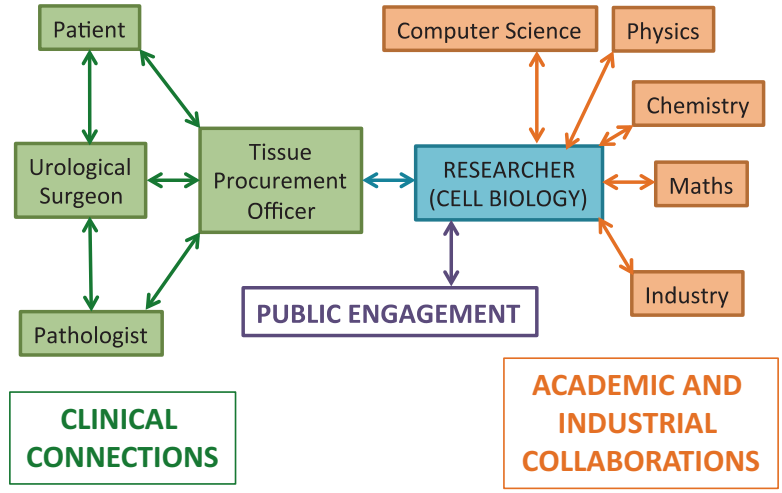

Fig. 2 Collaborative network: in order to establish clinical collaborations, several parties have to be invested and committed. There also has to be long-term continuity within the laboratory to maintain the links and the knowledge. Once the clinical connections are established, the tissue and cell resource attracts collaboration from other parties including academic departments and industry. The importance of the resource and the nature of the work can also be disseminated through public engagement

on primary cultures, there are two ways to assess their response: the first is to treat the whole population of cells and then separate out different cell types, and the second is to label cells with a fluorescent marker to be able to identify cells within the heterogeneous mixture. Both of these methods have their challenges; cell separation is a laborious procedure and it could be argued that cells change their behaviour when separate relative to when they are a mixture, and fluorescent labelling by whatever means always has the potential to change the cell behaviour. A new technique now available is trying to overcome both these challenges by using quantitative phase imaging (QPI) on heterogeneous cultures to observe individual cell response to drugs in real-time. The Livecyte™ is a microscope that uses the principle of ptychography to generate highly contrasted images such that several parameters can be measured for each cell. The measurements can be used to assess cell morphology (e.g., area and thickness sphericity), cell kinetics (e.g., velocity and meandering index) and population dynamics. For more detailed studies using this technology see these references [47–49]. The potential advantages of this technique are to establish cell signatures for different cell types and to identify rare outlier cells. Our previous studies using this technique showed that prostate cell lines and primary prostate epithelial cells differ in their size, speed and growth rates. We also showed that the transit amplifying cells (TA) and committed basal cells (CB) found within primary cultures have different cell signatures, with the TA cells being smaller, thicker and faster than the CB cells [27].

Typical drug treatment assays look at the average population, whether there is reduction in cell viability, death by apoptosis or effect on colony-forming ability. The rare cells that show resistance to therapeutic agent are likely to be the ones that don't fit in with the average and are therefore masked by their response. By treating every cell as a data-point, it is hoped that another layer can be peeled away and more information can be garnered that will contribute to the understanding of therapy resistance, tumour recurrence and identification of resistant cell features.

We carried out a study using primary prostate epithelial cells treated with docetaxel, a standard of care chemotherapy treatment for prostate cancer. Five concentrations were chosen to take a closer look at cell behaviour (Fig. 3). When looking at overall metrics, it became clear that as drug concentration increases, cell motility decreases,

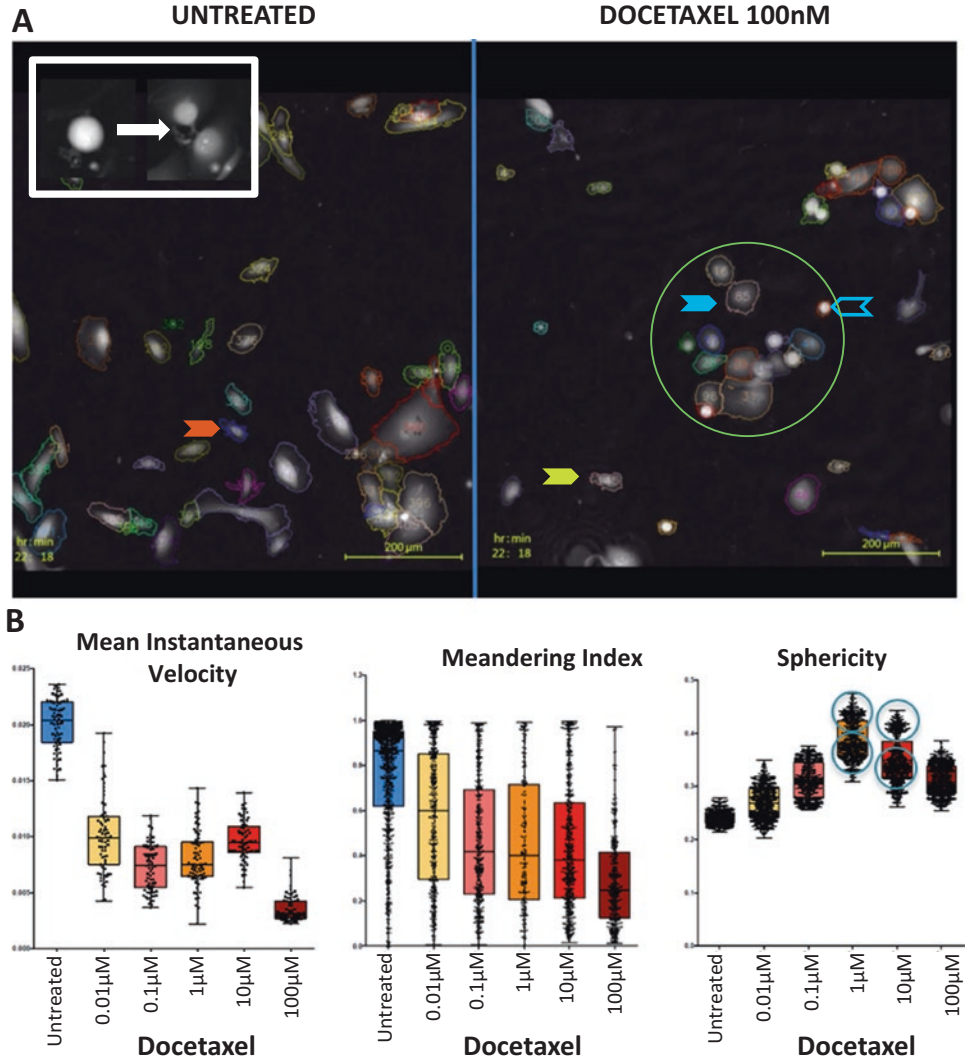

Fig. 3 Response of primary cells to docetaxel: (**a**) a time-lapse movie was generated over 72 h with images captured at 6 min intervals to observe the real-time response of primary prostate cells to docetaxel. Untreated cells could be seen moving and dividing in a continuous motion (red arrow). Dividing cell shown in white box insert. Treated cells predominantly responded by entering mitosis (cells rounding up—blue outline arrow) and when mitosis failed, due to the effect of docetaxel, the cells spread out and stopped moving (blue arrow). An outlier cell was observed that continuously rounded up to try to divide, but upon failure continued moving around in an erratic fashion (yellow arrow). (**b**) Kinetic and morphological features can be extracted from the data. Each cell is measured and patterns of response are recorded. Bimodal responses are observed with some parameters that can be related to the cell behaviour (turquoise circles). (Images were captured on a Livectye™ and data was analysed using the Cell Analysis Toolbox (CAT) software: www.phasefocus.com)

measured as cell velocity and meandering index. In addition, the sphericity of the cells increases with cells entering mitosis and not being able to divide due to the effect of the docetaxel [50]; this can clearly be seen in the video captured by the Livecyte™. Interestingly, when looking at the whole population, particularly at something like sphericity, a bi-modal or tri-modal response can be observed. Thus, it is necessary to closely observe the videos to identify unusual behaviour. One such cell is represented in Fig. 4. In comparison to the healthy cell that divides, and a cell

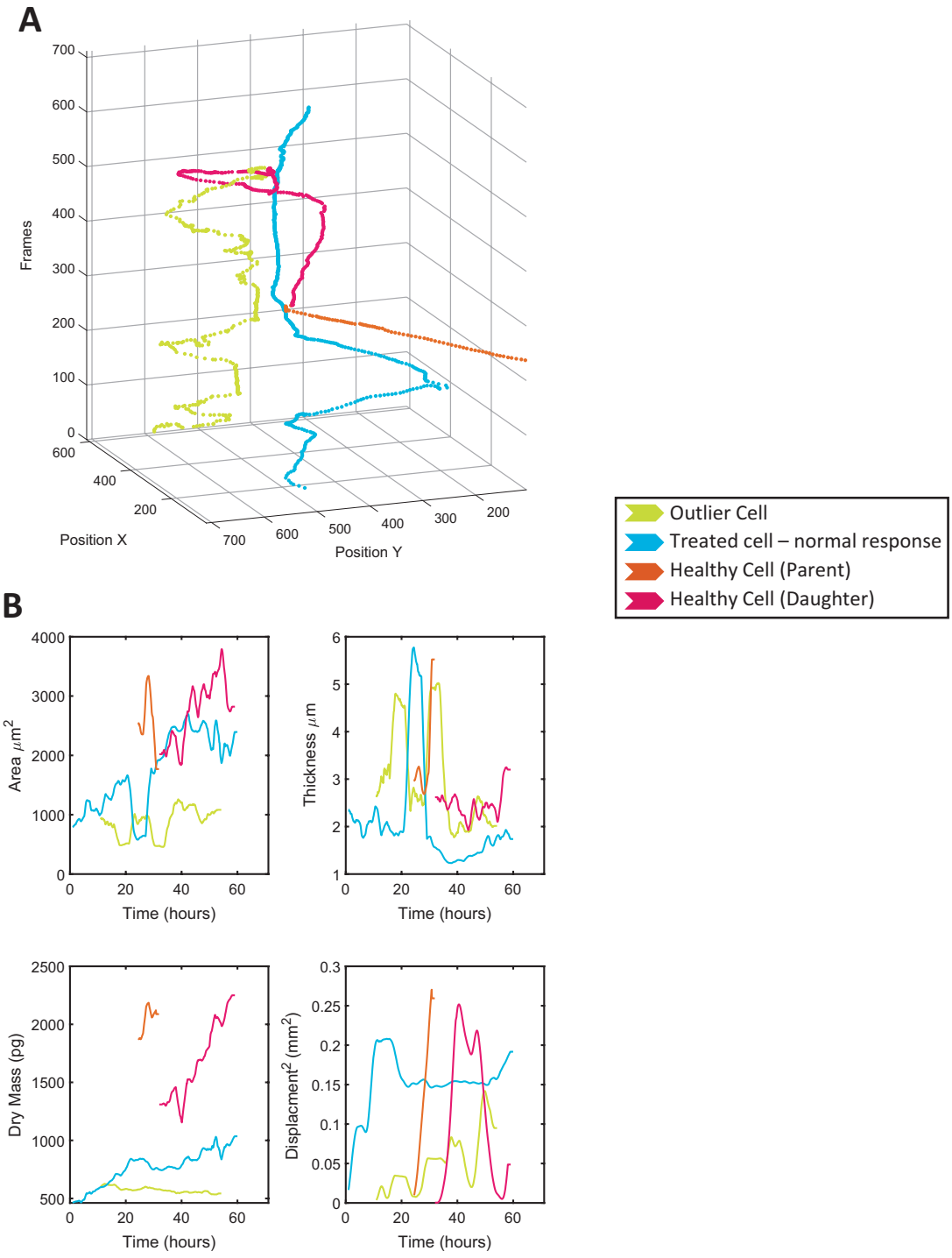

Fig. 4 Tracking individual cell behaviour: (**a**) Individual cells were tracked over time following response to docetaxel treatment (100 nM). Untreated (Healthy) cell has single direction of movement and then divides, and the daughter cell has meandering directionality. Typical cell response to docetaxel is to round up and enter mitosis, then following incomplete mitosis the cell stops moving. The outlier (putative resistant cell) is much more erratic in its behaviour and is very motile. Following attempted divisions, the cell maintains its highly motile behaviour. (**b**) Individual parameters also indicate the change in cell behaviour. Thickness clearly indicates the points at when the cell rounds up to enter mitosis

Table 1 When comparing disease state, epithelial hierarchy and therapeutic response a comprehensive cell signature should be considered

COMPREHENSIVE CELL SIGNATURE CHARACTERISTICS	COMPARISONS TO BE MADE		
Morphological Features	**Disease State**		
	Normal	v	Cancer
Cell Behaviour	**Epithelial Hierarchy**		
Kinetic Activity	Undifferentiated (progenitor) cells	v	Differentiated Cells
Gene Expression	**Response to Therapeutics**		
Protein Markers	Resistant Cells	v	Susceptible Cells

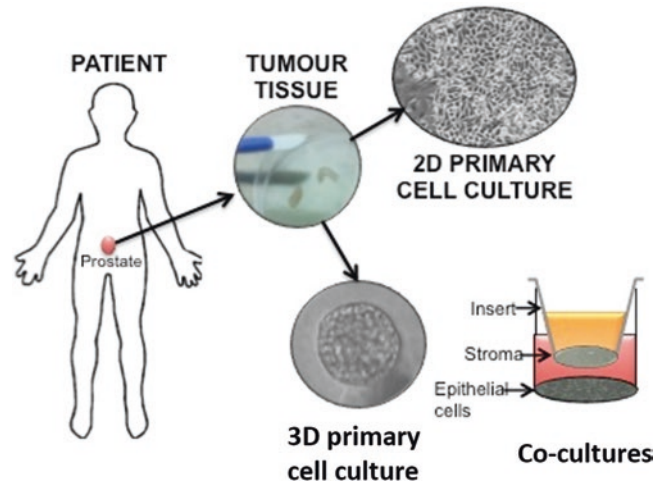

Fig. 5 Patient samples: primary prostate epithelial cells can be cultured from patient tissue. These cells can be grown in 2D, in co-culture systems and in 3D culture

that responds to drug in a typical way (failing to divide, spreading out and halting movement), we also show the outlier cell that is more erratic and sequentially rounds up, fails to divide and yet keeps moving. Only further analysis of outlier cells towards their ultimate fate will determine if the power of this new technique can be harnessed to identify resistant cell populations.

Discussion

Going forward, relationships between scientists, clinicians and patients are critical for the progression of prostate cancer research. When addressing treatment response and tumour recurrence, there are several levels of heterogeneity to take into account, which can only be done through use of patient material. Thus, the feasibility of having

primary cell cultures, which crucially represent modern disease, as a critical step in the lab to clinic pipeline, has to be explored. One successful example is the use of primary cells to develop an oncolytic adenovirus that is now in clinical trials [51, 52]. In order for this to become a broad reality, consistency, reproducibility, standardization and practicality are keys. The method of conditional reprogramming (CR) (using feeder cells and ROCK inhibitor) has been used by researchers in other cancer research fields and also by some researchers in the prostate field [53–55]. This is another method that requires further investigation and could be explored alongside the questioning of other fundamentals of tissue culture such as oxygen concentration; several studies have shown that cells can grow indefinitely in physiological oxygen concentrations of 2% and not the standard 20% [56–58].

The desire to have more consistent use of primary cells does not negate the need for useful cell lines, but it does highlight the need to explore the cell lines that already exist because there may already be the correct model to answer critical research questions. We also have to embrace new technology, to examine cell behaviour at another level of complexity. Combined with traditional markers, these technologies could help to give a more complete idea of individual cell signatures (Table 1).

Currently there are many methods that can translate patient prostate tissue to 2D cell culture, co-cultures with patient stroma and 3D models such as spheroids and organoids (Fig. 5). However, the range of media, matrices and apparatus that are used is vast [40, 59–67]. There is much work to be done if there is to be standardization across the prostate community globally, starting with the will to make it so.

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Role of Alternative Splicing in Prostate Cancer Aggressiveness and Drug Resistance in African Americans

Jacqueline Olender and Norman H. Lee

Abstract

Alternative splicing, the process of removing introns and joining exons of pre-mRNA, is critical for growth, development, tissue homeostasis, and species diversity. Dysregulation of alternative splicing can initiate and drive disease. Aberrant alternative splicing has been shown to promote the "hallmarks of cancer" in both hematological and solid cancers. Of interest, recent work has focused on the role of alternative splicing in prostate cancer and prostate cancer health disparities. We will provide a review of prostate cancer health disparities involving the African American population, alternative RNA splicing, and alternative splicing in prostate cancer. Lastly, we will summarize our work on differential alternative splicing in prostate cancer disparities and its implications for disparate health outcomes and therapeutic targets.

Keywords

Alternative splicing · Prostate cancer · Cancer disparities · Drug resistance

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Introduction

Alternative splicing of pre-mRNA plays a major role in both normal development and cancer progression. By hijacking and leveraging the complex and tightly regulated process of alternative splicing, cancer cells are able to acquire many of the "hallmarks of cancer" [1]. Prostate cancer (PCa), the most diagnosed cancer in men in the USA, is no exception. There have been several comprehensive review articles detailing the important role of alternative splicing in PCa progression and aggressiveness [1–7]. These reviews, however, do not address the critical topic of alternative splicing in PCa health disparities. PCa exhibits dramatic race/ethnic disparities as African American (AA) men have significantly higher risk, morbidity, and mortality compared to European American (EA) men. In this review, we will summarize some of the major molecular mechanisms and alternative splice events in PCa, as well as introduce our recent study elucidating the important role of differential alternative splicing in mediating PCa disparities.

Prostate Cancer Health Disparities

PCa is the most diagnosed cancer in men in the USA and accounts for over one-fifth of all newly diagnosed cancers in men [8]. More than 164,000 new cases are diagnosed each year, and PCa is

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the second leading cause of male-cancer-related deaths annually. PCa also has the highest heritability of any cancer at 10% [9]. In addition to family history, well-established risk factors of PCa include Lynch syndrome, age, and race/ethnicity [10, 11]. Despite increased screening and overall decreasing mortality rates of PCa, AA men have significantly higher rates of PCa incidence, high-risk cancer, and mortality [12]. AA men are 1.7 times more likely to be diagnosed with PCa and have a 2.4 times greater mortality rate compared to EA men [13]. This mortality ratio is the largest of any other malignancy in the USA [14]. Additionally, PCa appears to develop at an earlier age in AA men who present with significantly higher prostate-specific antigen (PSA) plasma levels, more clinically advanced disease, and develop higher grade metastatic disease at a three- to four-fold greater rate [15–18]. This health disparity has been attributed to epidemiological differences in socioeconomic status, health-seeking behavior, access to healthcare, and treatment plans [15, 16]. Even after adjusting for clinical and epidemiological factors, however, AA men still have significantly higher occurrence and mortality rates [19–21]. This disease disparity suggests that genetic ancestry plays an important role in PCa incidence, progression, and aggressiveness.

Molecular Differences in African American Prostate Cancer

Multiple studies have shown genetic and biological differences in prostate tumors in AA and EA patient populations. *TMPRSS2-ERG* gene fusions and *PTEN* deletions were once thought to be characteristic of all prostate tumors. However, recent reports have shown that these genetic alterations occur at a much lower frequency in AA PCa. Only 20–30% of AA PCa tumors contain *TMPRSS2-ERG* gene fusions compared to 40–50% in EA patients [22], and loss of *PTEN* was observed in 34% of EA men and only 18% of AAs [23].

Genome-wide association studies (GWAS) have identified multiple loci that confer a greater risk for PCa in AA men compared to EA men. The rs1447295 variant at the 8q24 locus has been associated with earlier diagnosis and increased risk in AA patients [24]. Six other variants (rs16901979, rs7000448, rs6983267, rs111906932, rs114798100, and rs111906923) have also been linked to increased PCa risk in AA men [25, 26]. African ancestry-specific PCa risk alleles have been identified at chromosomes 13q34 and 22q12 [27]. Additionally, a risk variant at the 17q21 locus has been found more frequently in men of African descent compared to other populations [28]. Many of these alleles reside within long coding RNA sequences.

Single nucleotide polymorphisms (SNPs) in genes that regulate androgen and testosterone metabolism have also been linked to PCa disparity in AAs. Polymorphisms in the cytochrome p450 enzyme *CYP17* increase the risk of PCa in AA men by 60% [29]. A homozygous "CC" genotype in the 5′ promoter region (rs743572) in AA men is clinically associated with advanced PCa disease [30].

In terms of the cancer transcriptome, AA PCa has been shown to exhibit increased expression of genes that promote growth (e.g., *EGFR* and *AKT1*) and metastasis (e.g., *CXCR4* and *BMP2*) compared to EA PCa [31–33]. For the *IL-6* gene, a race-specific and anti-correlated expression pattern is observed during PCa progression. Namely, EA PCa has increased expression of *IL-6* compared to EA normal prostate, while *IL-6* is downregulated in AA PCa compared to AA normal prostate [34]. Exogenous treatment with IL-6 downregulated TP53 in AA PCa cell lines and upregulated expression of a splice variant of *MBD2*, promoting a cancer stem-like cell phenotype [34]. Additionally, AA PCa exhibits an increased inflammatory signature, including increased expression of inflammatory genes (e.g., *CCR7*) and more frequent copy number variations of genes related to the immune response (e.g., *IL-27*, *ITGAL*, and *ITGAM*) [31, 33, 35, 36].

AA PCa cell lines and patient specimens have distinct miRNA profiles compared to EA PCa. AA PCa cell lines have increased expression of hsa-miR-26a compared to EA cell lines derived

from tumors of similar stage and grade [37]. Theodore et al. [38] showed decreased expression of five miRNAs due to hypermethylation of CpG islands within promoter regions in AA PCa. Of particular interest, miR-152 had significantly lower expression in AA patients versus EA patients (in both non- and malignant tissue). Ectopic over-expression of miR-152 in PCa cell lines downregulated expression of DNMT1 by binding to the 3′UTR of the mRNA, leading to decreased proliferation, migration, and invasion.

Ten miRNAs have been identified that exhibit enriched or depleted expression in AA versus EA PCa [39]. These miRNAs, including miR-133a (AA depleted), miR-513c (AA depleted), and miR-96 (AA enriched), were computationally predicted and experimentally shown to target key genes known to promote cancer, such as *MCL1, STAT1*, and *FOXO3A*. Ectopic treatment of PCa cell lines with AA-depleted miRNA mimics (for miR-133a and -513c) or AA-enriched miRNA antagomirs (for miR-96) resulted in decreased proliferation, invasion, and caspase activity. In agreement with these in vitro findings, AA PCa specimens showed significantly increased expression of MCL-1 and STAT1 and decreased expression of FOXO3A compared to EA PCa samples.

The role of epigenetics in PCa disparities is also being explored. Using quantitative pyrosequencing, Kwabi-Addo et al. [40] and Devaney et al. [41] revealed increased gene promoter methylation in AA PCa specimens compared to EA PCa. *RARβ2*, *SPARC*, *TIMP3*, *NKXX2-5*, *ABCG5*, and *SNRPN* genes were all found to be highly methylated in AA PCa samples and cell lines. Tang et al. [42] identified an association between increased *RARB* and *APC* methylation and increased PCa risk in AA men.

Alternative Splicing

An area of research that has recently garnered considerable attention with the advent of genome-wide approaches (e.g., exon arrays and RNA-Seq) is the role of alternative splicing (AS) in cancer and cancer disparities. AS is the major mechanism for post-transcriptional regulation of gene expression, mRNA diversity, and protein modification. During AS, introns are typically excised from the precursor mRNA (pre-mRNA) and the remaining exons can be joined together in different combinations to produce multiple unique mature mRNA transcripts from a single gene. It is estimated that over 90% of human genes transcribe pre-mRNAs that undergo AS with an average of five unique mRNA variants per coding gene. This generates a proteomic complexity of ~100,000 distinct protein isoforms from ~20,000 protein-coding genes. Types of splicing events include exon skipping (removal of specific exons), cryptic exon expression, selection between two mutually exclusive exons, exon scrambling, intron retention, alternative 5′ or 3′ splice sites (altering boundaries between introns and exons), alternative promoters (which can alter reading frames), and alternative polyadenylation sites (Fig. 1). This is a highly complex and flexible system that responds to cell type, tissue type, developmental stage, physiological system, and disease state.

AS generates a variety of protein isoforms with different sequences and altered functions from the same gene, promoting diversification of the transcriptome and proteome at both the species and interspecies levels. Although not all AS variants are functional, many can have similar or different functions, different stability kinetics, alternative subcellular localizations, or encode isoforms that are susceptible to different post-translational modifications (e.g., phosphorylation and ubiquitination). By altering the repertoire of splice variants within a cell in a time- and/or spatial-dependent manner, AS can lead to protein isoforms with different interactome networks by promoting or inhibiting different DNA–protein, protein–protein, protein–ligand, and protein–drug interactions.

Splicing events are regulated by *cis*-acting sequences (splice sites, splicing enhancers or silencers, and branch points) located within the pre-mRNA and 30–500 *trans*-acting factors of the spliceosome, including small nuclear RNAs (snRNAs) and RNA-binding proteins (RBPs). AS is also strongly influenced by RNA polymerase kinetics, chromatin modifications,

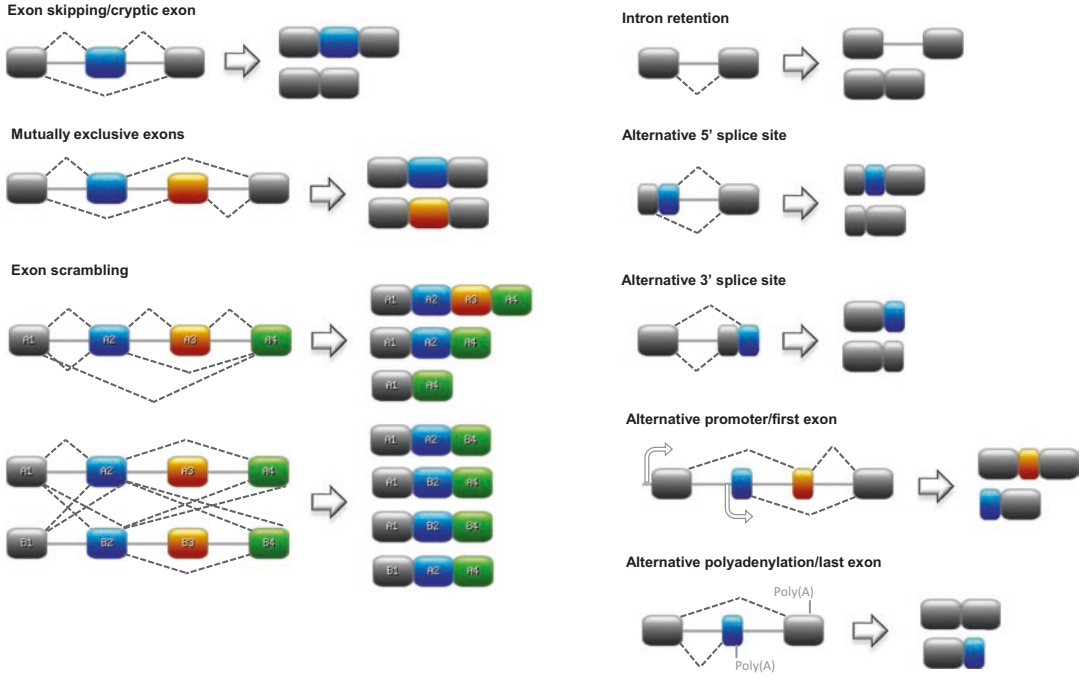

Fig. 1 Schematic representations of different types of splicing events. Exons are depicted as rectangles and introns as solid lines. Broken lines represent splicing events. Abbreviations: Poly(A), polyadenylation site. Designed on https://prosite.expasy.org

chromatin structure, epigenetic modifications (e.g., DNA and/or RNA methylation), nucleosome occupancy, location of *cis*-elements, secondary structure of pre-mRNA, and sequence editing [43, 44].

Cis-regulatory sequences are divided into two groups: splice sites that are required for spliceosome binding and binding sites for other RBPs. Sequences within exons (5′ and 3′ splice sites) and within introns (branch point and polypyrimidine sequences) designate exon–intron boundaries for the spliceosome. These splice sites can be constitutive (always recognized as splice sites) or alternative. The strength of a splice site is important for splicing accuracy and frequency. Strong splice sites contain consensus sequences that are well recognized by the spliceosome, thereby undergo splicing at a high rate. Weak splice sites rely on *cis*-acting sequences and cell context for splicing to occur. Splicing regulatory elements (SREs) include intronic or exonic splicing enhancers (ISE, ESE) or silencers (ISS, ESS). These provide binding sites for *trans*-acting factors, such as splicing factors (SF).

The spliceosome is composed of five snRNPs and over 200 SFs and auxiliary proteins. SnRPs (U1, U2, U4, U5, and U6) are the core components of the spliceosome ribozyme and are responsible for recognizing splice sites. The spliceosome also contains DEAD/H-box RNA-dependent ATPases that allow for changes in RNA–RNA base pairing [45]. A splicing event begins with U1 binding, the 5′ splice site, the SF3b complex within U2 binding, the branch point site, and U2AF1 and U2AF2 auxiliary proteins binding, the 3′ splice site. U1 and U2 interact to form the pre-spliceosome. Next, U4, 5, and 6 are recruited, the spliceosome rearranges, U1 and U4 are released, and the spliceosome becomes activated. In the first splicing reaction, the phosphodiester bond at the 5′ splice site is cleaved via nucleophilic attack from the adenosine in the branch point site. The intron then forms an intermediate lariat structure, and the

phosphodiester bond at the 3′ splice site is cleaved via nucleophilic attack by the free 3′ hydroxyl group on the phosphate of the 3′ splice site. Finally, the two exons are ligated together and the intron lariat is released [46].

Trans-acting RBPs, such as SFs and auxiliary proteins (e.g., SF1 and U2AF), complex with the spliceosome to add additional flexibility and complexity to the splicing process. RBPs bind *cis*-regulatory sites to promote or inhibit splice site recognition which is dependent on location of binding (e.g., within intronic or exonic sequences, upstream of an alternative exon, and within a downstream intron), cellular context, regulation by other RBPs, and expression level of the RBP [47, 48]. The most well-studied *trans*-acting factors are the serine/arginine-rich SF (SRSF) and heterogeneous nuclear ribonucleoprotein particle (hnRNP) families. SRSFs are composed of two RNA recognition motifs at the N-terminus and a serine-rich domain at the C-terminus that is involved in protein–protein interactions. SRSFs are generally considered positive splicing regulators. They promote exon inclusion by preferentially binding to purine-rich ESE or ISE sequences and recruiting U1 to 5′ splice sites and U2AF to 3′ splice sites [49]. SRSF protein kinases (SRPKs) and CDC-like kinases (CLKs) activate SRSFs by phosphorylation in the cytoplasm or nucleus, respectively. The hnRNP family is largely classified as negative splicing regulators. Like SRSFs, they have two RNA recognition motifs; however, their protein–protein interaction domains are unstructured. HnRNPs promote exon skipping by binding ESS and ISS sequences and inhibiting recognition of splice sites. They may also prevent spliceosome assembly after 3′ splice site recognition via steric hindrance of snRNPs.

SRSFs and hnRNPs have more nuanced roles than exclusively positive or negative splicing regulators [50, 51]. Their effect on splicing can depend on several factors, such as the location of the binding site. For example, SRSFs enhance splicing when binding to sequences within exons and repress splicing when bound to introns [49]. The functional consequences of SF binding can also be influenced by cell differentiation, cell fate, tissue identity, organ development, and disease state [52].

Alternative Splicing and Cancer

All components of the splicing process are tightly regulated, and any alteration can lead to disease causation and progression. The involvement of splicing dysregulation in oncogenic processes is known to activate oncogenes and inactivate tumor suppressors. Gene expression program changes via aberrant splicing in cancer cells select for functional changes that promote the malignant progression of the tumor [53]. Modifications in the splice sites or splicing machinery can lead to DNA damage, genomic instability, changes in epigenetics, alterations in transcriptional elongation, and changes in gene expression, thus helping to promote any of the "hallmarks of cancer" [54–56]. Splice variants are being used to characterize tumor subtypes and are targets of interest for cancer biomarkers and therapeutics [57]. Due to the potential for functional differences, individual AS variants need to be studied separately to better understand each variant's role in disease progression. In addition, an understanding of the overall splicing changes, as a change in one *trans*-acting factor can affect the splicing of hundreds of transcripts, will be instrumental in identifying the role of AS in cancer.

The Cancer Genome Atlas (TCGA) data have been used to identify genome-wide AS events in cancer versus normal tissues and between different tumor subtypes and stages. Globally, AS events occur more frequently than somatic mutations in driver genes. AS also occurs more often in cancer-related pathways and in genes that are frequently mutated in cancers [58, 59]. Analysis of TCGA data has also identified key somatic mutations in splice sites that affect exon–intron boundaries, resulting in changes in expression of oncogenes and tumor suppressors in cancer [60]. In general, splicing of proto-oncogenes generates constitutively active or gain of function variants that confer an increased oncogenic advantage. Synonymous mutations, which can alter splice sites, are also more highly enriched in oncogenes.

Conversely, AS of tumor suppressors can introduce premature stop codons and altered reading frames, resulting in decreased protein levels via nonsense-mediated decay or decreased function. Cancer cells have increased levels of intron retention in tumor suppressor transcripts which promote premature termination, nonsense-mediated decay, and tumor suppressor inactivation [61–63]. Mutations in splice sites or splice site choice can result in isoform switching or generation of novel splice variants [64]. Thus, somatic mutations in key genes or splice sites involved in AS may be a major driver in many cancers.

Differential splicing can generate variants with opposing functions or shift the balance between two isoforms. For example, while the full-length isoform of caspase-9 is pro-apoptotic, a shorter isoform missing exons 3–6 is anti-apoptotic and has been identified in cancers, including non-small cell lung carcinoma [65]. SRSF1, which is overexpressed in many cancers, binds within intron 6 to promote inclusion of exons 3–6 to generate the long variant [66]. Conversely, hnRNPL binds an ESS in exon 3 and induces splicing exclusion of exons 3–6 to generate the short variant [67]. Kinases such as AKT are predicted to phosphorylate and activate both SRSF1 and hnRNPL [68]. The known tumor suppressor gene *TP53* has over seven different splice variants that have been detected in a variety of cancers [69]. Splicing events are concentrated in the 5′ and 3′ ends and result in alternative promoter selection, exon skipping, intron retention, alternative 5′ and 3′ splice sites, or alternative reading frames. These P53 isoforms can inhibit full-length P53, impair growth or senescence suppression, and are associated with decreased patient survival.

Frequently, tumors display alterations in *trans*-acting factors. Perturbations in the expression level, localization, activity, or degradation of RBPs, SFs, or their upstream regulators can vary dramatically between different cancers. While hnRNPA2/B1 is an oncogenic driver in glioblastoma via splicing of tumor suppressors *IG20* and *MST1R* (*RON*) [70], RBM4 controls apoptosis, proliferation, and migration as a tumor suppressor in a variety of other solid tumors [71]. The most common SF mutations in hematological and solid tumors are heterozygous missense gain or alteration of function mutations in *SF3B1*, *U2AF1*, and *SRSF2* and homozygous loss of function mutations in *ZRSR2* [54, 72]. SF3B1 is a member of the SF3b complex within the U2 snRNP of the spliceosome. Mutations have been observed in the 3′ splice site of the *SF3B1* pre-mRNA resulting in nonsense-mediated decay, which commonly occurs in breast cancer [73]. Mutations in the zinc finger domains of *U2AF1*, a U2 small nuclear RNA auxiliary factor, are frequently identified in non-small cell lung cancer [74]. Missense mutations are often observed in *SRSF2* that change its binding affinity to ESE sequences and are common in chronic myelomonocytic leukemia [75]. ZRSR2 is a zinc finger RBP in the U12 minor spliceosome complex. Mutations that introduce in-frame stop codons or disrupt the reading frame are common in myelodysplastic syndrome [76].

Due to the pivotal role of AS in cancer, many researchers are focusing their efforts on identifying or developing molecules that target aberrant AS in cancers [44, 54, 77]. Potential targets for these therapies include mutations in splice sites, *cis*-regulatory elements, and promoter or coding regions of *trans*-acting factors. Due to the high mutation rates observed in cancers, SF3B1 is a major target for splicing-modulating drugs. In addition, upstream factors such as SF kinases and specific splice isoforms of oncogenes or tumor suppressors are also attractive targets. Cancers that rely on splicing activity are ideal candidates for AS-targeted therapy. For example, MYC-driven cancers rely on the spliceosome, through BUD31, for promoting oncogenesis [78]. BUD31 associates with SF3B1, U2AF1, and other core spliceosome factors. Inhibition of the spliceosome via spliceosome inhibitors or *BUD31* depletion downregulates survival, tumor growth, and metastatic potential of breast cancers driven by MYC.

There are a variety of types of therapeutic compounds used to target AS. The most well-known are antisense oligonucleotides (ASOs) which are composed of nucleotides or analogs that hybridize with a complimentary nucleic acid

sequence. By coding for the complimentary sequence of the target, ASOs can potentially block splice sites via steric hindrance, target mRNAs for degradation, redirect splicing, or prevent *trans*-factors from binding. ASOs have gained traction in treating Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA), and amyotrophic lateral sclerosis (ALS) [44]. In oncology, two ASOs, AZD9150 targeting *STAT3* and AZD4785 targeting *KRAS*, are in clinical trials for solid advanced and metastatic diseases [79, 80].

Small molecule inhibitors (SMIs) have been designed to target SF kinases and spliceosome components. SRPIN340 which targets SRPKs and TG-003 which targets CLKs cause decreased activity of SFs and subsequent decreased expression of "splice-correct" signaling proteins, such as VEGF and p70-S6K [81, 82]. ML315, a chemically modified quinazoline probe, selectively inhibits the CLK family as well [83]. Cp028 has been shown to inhibit intermediate stage spliceosome assembly by causing the early release of U4/U6 [84].

Natural products and their derivatives have also shown promise in targeting different stages of AS. Leucettine L_{41}, derived from the marine sponge product leucettamine B, is an ATP-competitive inhibitor against CLK1 and CLK3 and has been shown to inhibit phosphorylation of SRSF4 and SRSF6 [85]. Another natural product, *N*-palmitoyl-l-leucine, targets late stage spliceosome assembly [86].

Indole derivatives, such as benzopyridoindoles and pyridocarbazoles, alter SF-ESE-dependent splicing in key oncogenic genes such as *MST1R* [87, 88]. The RBP RBM39 is a target of sulfonamides derived from para-aminobenzoic. Mutations in RBM39 and resistance to indisulam are common in leukemia and lymphomas. These mutations block the complex formation of RBM39 with the CUL4-DCAF15 ubiquitin ligase complex, halting the normal proteasomal degradation of RBM39 and resulting in aberrant pre-mRNA splicing [89]. SRPIN340 is an isonicotinamide compound shown to inhibit expression of SRPK1 and a pro-angiogenic *VEGF* variant [90]. Derivatives of the natural compound FR901464 have shown promising ability to inhibit SF3B. These analogs include spliceostatin A, meayamycin, and sudemycin [91–93].

Pladienolide-scaffold derivatives have had the most success in clinical trials. E7107, derived from pladienolide B, inhibits SAP130 of the SF3B complex [94]. This weakens the binding interaction between U2 and the pre-mRNA by locking SF3B1 in an inactive conformation and sterically preventing binding to the branch point adenosine [95]. E7107 was one of the first splicing modulator drugs to enter clinical trials in solid tumors in 2007 [96, 97]; however, further studies in humans were suspended due to unexpected toxicity. H3B-8800, another pladienolide derivative, selectively inhibits wild-type and mutated SF3B1 isoforms and enriches for intron retention in SF-coding mRNAs [98]. Trials using H3B-8800 in hematological cancers have been ongoing since 2016.

Our current limited understanding of overall "splicing sickness," restoration of normal splicing, and downstream effects of spliceosomal mutations need to be addressed in order to develop new AS drugs. Overcoming issues of systemic delivery, toxicity, off-target effects, efficacy, and targeting the desired cell type will be keys in splice-modulating therapies, becoming a safe and efficacious therapeutic option for cancer patients.

Alternative Splicing in Prostate Cancer

A number of genes undergoing AS have been associated with PCa development and progression. The androgen receptor (AR), a steroid nuclear hormone that plays a major role in normal prostate homeostasis and PCa development, is the primary target for early PCa treatment. PCa tumors, however, develop AR-targeted treatment resistance (i.e., castrate-resistant) as the disease progresses. One mechanism in which PCa tumors develop drug resistance is through AS of the *AR*. Among the 20 different *AR* splice variants identified, *ARv7* is the most clinically frequent and relevant variant. The *ARv7* variant is generated by

inclusion of a cryptic exon within exon 3 that encodes a protein isoform with a truncation of the entire C-terminal ligand binding domain (LBD). The LBD is important for AR activation by androgens and subsequent translocation of the AR into the nucleus for transcriptional regulation of androgen-dependent genes. The ARv7 isoform acts independently of androgen binding and is constitutively present in the nucleus of prostate cells, regardless of androgen stimulation [99]. Levels of *ARv7* mRNA in PCa patients can help predict responsiveness to anti-androgen therapies, such as abiraterone and enzalutamide [100]. The SF hnRNPA1 and RBP SAM68 are believed to contribute to the regulation of the *ARv7* variant. Relocalization of hnRNPA1 from the nucleus to the cytoplasm decreases expression of *ARv7* in PCa cells and resensitizes them to enzalutamide [100–103]. SAM68 preferentially increases expression of ARv7 in PCa cells, via SAM68 stabilization of the *ARv7* mRNA via direct RNA–protein binding and indirect mediation by SRSF1 [104].

A second clinically relevant *AR* splice variant, *ARv567es*, has been identified in PCa cells where exons 5–7 (of 8 total) are skipped, truncating the majority of the LBD. Similar to the ARv7 isoform, ARv567es is constitutively active and androgen independent [105]. This variant is highly expressed in metastatic and malignant prostate tissue [106]. ARv567es regulates oncogenes involved in cell cycle progression including *UBE2C*, which codes for a ubiquitin-conjugating protein involved in the machinery that inactivates the mitotic checkpoint and promotes proliferation [107].

The *fibroblast growth factor receptor* (*FGFR*) *2* undergoes AS of the third Ig-like extracellular domain, generating two isoforms: FGFR2IIIb and FGFR2IIIc. FGFR2IIIb is expressed highly in normal prostate epithelial cells and is a known tumor suppressor. FGFR2IIIc is involved in autocrine signaling and expressed more highly in mesenchymal cells. While no change in overall FGFR2 protein expression is observed as PCa progresses [108], a switch in FGFR2 isoforms occurs due to AS. Decreased expression of the IIIb isoform and exclusive expression of the IIIc isoform is associated with epithelial to mesenchyme transition (EMT) and loss of AR sensitivity [109]. This increase in FGFR2IIIc expression correlates with an increase in fibroblast growth factor (FGF) 8b, a ligand associated with PCa [110].

The vascular endothelial growth factor (VEGF) is largely responsible for cellular growth and survival via angiogenesis in both normal and cancerous conditions. “Canonical” splicing of *VEGF* produces a VEGF isoform that is pro-angiogenic, while an alternative 3′ splice site event generates an anti-angiogenic isoform $VEGF_{165}b$, which is the main isoform. $VEGF_{165}b$ differs from pro-angiogenic VEGF in the last six amino acids and acts as an antagonist of the VEGF receptor [111]. Expression of pro-angiogenic VEGF is an early driver of PCa, and increased expression corresponds with later stage PCa and increased expression of SRSF1 [112]. Inhibition of the SF kinase SRPK1, a known activator of SRSF1, causes splice switching of $VEGF_{165}b$ in PCa cells and decreased tumor formation in PCa mouse models [113].

Bcl-x plays a pivotal role in regulating apoptosis. Alternative 5′ splice site usage within exon 2 of the *BCL2L1* pre-mRNA generates two variants that have opposing functions. The long anti-apoptotic isoform, Bcl-x(L), is associated with cell survival, while the shorter isoform Bcl-x(S) promotes apoptosis, cell death, and sensitivity to chemotherapeutics in PCa [114]. High Bcl-x(L) to Bcl-x(S) ratios have been observed in PCa and over-expression of the short isoform induces apoptosis-mediated cell death in cancer cells [1, 6]. SAM68 selectively favors the upstream 5′ splice site, thus favoring production of the *BCL2L1* long variant and preventing apoptosis. Increased expression of Bcl-x(L) has been identified in PCa patients and cell lines, resulting in decreased apoptotic-induced cell death and decreased sensitivity to cytotoxic therapeutics [115].

Splice variants of another apoptotic-related gene *SH3GLB1*, which codes for the BAX-binding protein Bif-1, has recently been implicated in the transition of adenocarcinoma to aggressive treatment-induced neuroendocrine

(t-NE) PCa. Bif-1a, the pro-apoptotic protein isoform encoded by a variant lacking exon 6, is the predominant isoform expressed in adenocarcinoma specimens [116]. Bif-1b (encoded by a variant containing a short version of exon 6) and Bif-1c (encoded by a variant containing a long version of exon 6), however, become highly expressed in t-NE PCa. This switch in dominant variant expression is regulated by the SF SRRM4.

Cyclin D1 (CCDN1) associates with cyclin-dependent kinase 4 (CDK4) to promote cell cycle progression through the G1 phase. Two alternative splice variants of *CCDN1* have been identified: the cyclin D1a mRNA, which is the full-length and more common variant, and the cyclin D1b variant, in which intron 4 is retained leading to early termination. The cyclin D1b protein isoform plays a distinct role as an AR co-regulator to promote expression of AR-dependent genes associated with tumor growth and metastasis in PCa, specifically *SNAI1* [117]. Additionally, increased expression of SRSF1 in PCa cells correlates with enhanced expression of cyclin D1b, but not D1a [118, 119].

ST6GalNac1 is an enzyme that synthesizes the sialyl-T (sTn) antigen and modifies the glycosylation pattern of cell surface glycoproteins that play a role in cell adhesion and metastasis. ST6GalNac1 is androgen-sensitive, thus indicating a role for this enzyme in PCa. Recently, RNA-seq data have identified a shorter splice variant of *ST6GalNac1* that has only been reported in PCa [120]. The short isoform results from the inclusion of an additional exon (exon 2) within the 5′ UTR that generates a new start codon and encodes a longer mRNA variant but a shorter, fully functional protein isoform that has increased expression compared to the full-length protein isoform missing exon 2. In vitro studies suggest a role for the short isoform in promoting EMT through decreased cell adhesion and increased cell motility.

A new splice variant of *PCSK6* has been identified in PCa. *PCSK6* codes for the proprotein convertase PACE4 that modifies proprotein substrates in secretory and known oncogenic pathways. Couture et al. [121] identified a variant of *PCSK6* with a shorter 3′UTR via AS of exon 25 (variant known as *PACE4-altCT*). While both the full-length PACE4 and the shorter PACE4-altCT are expressed in PCa specimens, PACE4-altCT showed increased expression in higher grade tumors. Additionally, PACE4-altCT appears less susceptible to degradation and secretion, is more stable, more rapidly activated, and increases growth and proliferation when compared to the full-length protein. PACE4-altCT directly increases the processing of pro-GDF15 (i.e., prostate differentiation factor), a TGFβ ligand with a known role in immunosuppression, protection against radiation-induced cell death, and neovascularization.

Three splice variants of *CLK1* have been identified: full-length *CLK1*, $CLK1^{T1}$ (skipping of exon 4), and $CLK1^{T2}$ (retention of intron 4). CLK1 is responsible for phosphorylating and activating SRSFs and other SFs. Both T1 and T2 isoforms lack the catalytic domain and are inactive. The $CLK1^{T2}$ has been found to be the more prominent isoform in PCa. Treating PCa cells with a CLK1 inhibitor shifts the *CLK1* variant expression ratio to favor both the expression of full-length active CLK1, as well as expression of the pro-apoptotic variants of *CASP9, MCL-1, BCL2L1*, and *survivin* [122].

The *HSD17B4* gene encodes 17β-hydroxysteroid dehydrogenase type 4 (17βHSD4), an enzyme involved in testosterone and dihydrotestosterone metabolism. Recently, five splice variants of *HSD17B4* were identified, four of which encode enzyme isoforms that do not inactivate testosterone and dihydrotestosterone via conversion to inert steroid products [123]. The remaining isoform, isoform 2, is the major enzyme expressed in prostate tissue and is able to inactivate androgens. The splice variant encoding isoform 2 of 17βHSD4 is missing part of exon 2 and all of exon 3, which code for sections of the short-chain alcohol dehydrogenase domain. This isoform was found to be functionally suppressed in metastatic castration-resistant PCa.

As outlined above, AS plays an important role in PCa development, progression, and drug resistance. While it is fairly well accepted that AA PCa is genetically different from EA PCa, the role of AS in PCa disparities is less clear. Over

Table 1 Summary of splicing events in prostate cancer

Reference	Spliced gene (splicing factor)	Alternative splicing event	Cell lines and/or patient samples
Cao et al. [99]	*ARV7*	Cryptic exon	LNCaP, **22Rv1**, PC-3
Antonarakis et al. [100]	*ARV7* (hnRNPA1)	Cryptic exon	Patients (ethnicity N/A)
Ko et al. [101]	*ARV7* (hnRNPA1)	Cryptic exon	PC-3
Nadiminty et al. [102]	*ARV7* (hnRNPA1)	Cryptic exon	LNCaP, **22Rv1**, VCaP, C4-2B
Tummala et al. [103]	*ARV7* (hnRNPA1)	Cryptic exon	**22Rv1**, C4-2B
Stockley et al. [104]	*ARV7* (Sam68, SRSF1)	Cryptic exon	**22Rv1**, VCaP, CWR22, PC3-M
Sun et al. [105]	*ARv567es*	Exon skipping	**M12**, LNCaP, LuCaP
Hörnberg et al. [106]	*ARv567es*	Exon skipping	Patients (ethnicity N/A)
Liu et al. [107]	*ARv567es*(MED1)	Exon skipping	LNCaP, **M12**
Sahadevan et al. [108]	*FGFR2*	Mutually exclusive exons	PC-3, DU145, patients (ethnicity N/A)
Carstens et al. [109]	*FGFR2*	Mutually exclusive exons	LNCaP,PC-3, DU145, DUP9479, DUKAP-1, DUPKAP-2
Gnanapragasam et al. [110]	*FGFR2*	Mutually exclusive exons	Patients (ethnicity N/A)
Woolard et al. [111]	*VEGF*	Alternative 3′ splice site	Patients (ethnicity N/A)
Rennel et al. [112]	*VEGF*	Alternative 3′ splice site	PC-3, patients (ethnicity N/A)
Mavrou et al. [113]	*VEGF*	Alternative 3′ splice site	PC3, LNCaP, DU145
Mercatante et al. [114]	*BCL2L1*	Alternative 5′ splice site	PC-3
Busà et al. [115]	*BCL2L1* (SAM68)	Alternative 5′ splice site	LNCaP, patients (ethnicity N/A)
Gan et al. [116]	*SH3GLB1* (SRRM4)	Alternative 5′ splice site, exon skipping	LNCaP, **22RV1**, PC-3, DU145, NCI-H660, LNCaP95, patients (ethnicity N/A)
Augello et al. [117]	*CCND1*	Intron retention	LNCaP, VCaP, LAPC4, PC-3, C4-2
Olshavsky et al. [118]	*CCND1* (SRSF1)	Intron retention	LNCaP, LAPC4
Paronetto et al. [119]	*CCND1*	Intron retention	PC3, LNCaP
Munkley et al. [120]	*ST6GalNAc1*	Exon skipping	LNCaP, VCaP, PC-3, 22Rv1, DU145, LNCaP-AI, and LNCaP-cdxR, BPH-1
Couture et al. [121]	*PCSK6*	Exon skipping	DU145, LNCaP, patients (ethnicity N/A)
Uzor et al. [122]	*CLK1*	Exon skipping, intron retention	PC-3, DU145, VCaP
Ko et al. [123]	*17βHSD4*	Alternative 5′ splice site, exon skipping	LNCaP, LAPC4, VCaP, PC-3, DU145, RWPE-1, **22Rv1**, patients (ethnicity N/A)
Wang et al. [59]	*PIK3CD*	Exon skipping	**MDA PCa 2b**, VCaP, LNCaP, PC-3, **patients** (AA, EA)

Cell lines and patient samples with known African American ancestry are in bold and underlined
ARV7 androgen receptor splice variant 7, *hnRNPA1* heterogeneous nuclear ribonucleoprotein A1, *SRSF1* serine/arginine splicing factor 1, ARv567es androgen receptor variant (exons) 5,6,7 exon skipping, *MED1* mediator complex subunit 1, *FGFR2* fibroblast growth factor receptor 2, *VEGF* vascular endothelial growth factor, *BCL2L1* B-cell lymphoma 2-like 1, *Sam68* Src-associated substrate in mitosis of 68 kDa, *SH3GLB1* SH3 domain-containing GRB2-like protein B1, *SRRM4* serine/arginine repetitive matrix 4, *CCND1* cyclin D1, *SRSF1* serine/arginine splicing factor 1, *ST6GalNAc1* ST6 *N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1, *PCSK6*, proprotein convertase subtilisin/kexin type 6, *CLK1* CDC-like kinase 1, *17βHSD4* 17β-Hydroxysteroid dehydrogenase type 4, *PIK3CD* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta, *AA* African American, *EA* European American

60% of the studies cited above used only cell lines derived from EA patients (Table 1). Of the remaining studies, two cell lines (22RV1 and M12) of mixed or self-reported AA ancestry were utilized. The 22RV1 cell line was derived from the CWR22 line, a primary prostatic carcinoma serially transplanted in nude mice [124, 125]. A recent study determined 22RV1 was only 41% AA ancestry [126]. The M12 line was immortalized from the P69SV40T cell line via transfection with SV40 T antigen and passaged in nude mice [127, 128]. While the ancestry of the M12 line has not been confirmed by genotyping, the parental cell line was reported to be derived from prostate epithelial cells from a 63-year-old AA man. None of these studies use an AA PCa cell line with over 50% AA genetic ancestry, such as MDA PCa 2b or RC77 T/E (74% and 73%, respectively) [126]. Forty-one percent of the studies cited analyzed primary prostatic samples, but none specified the ancestry (genotyped or self-reported) of the patients.

The lack of AA cell lines and patient samples used in AS PCa studies reflects the lack of minority subjects across all cancer, and specifically PCa, research [129]. In order to better understand PCa disparities, eliminate the disproportionate disease burden, provide novel biomarkers, and improve survival and quality of life in AA PCa patients, we must increase the use of AA PCa cell lines and specimens in our research.

Differential Alternative Splicing of *PIK3CD* in Prostate Cancer Disparities

In order to further our understanding of AS in PCa health disparities, we applied a functional genomics approach to investigate differential AS (dAS) events in AA and EA PCa patients [59]. Twenty AA and 15 EA PCa tumor and matched normal specimens (treatment naïve, Gleason score 6–8, age 49–81) were collected, and samples were analyzed using the Affymetrix Human GeneChip exon array to identify genes undergoing AS (Table 2). We identified 158 unique genes that underwent AS in both AA and EA PCa. It can be concluded that these genes, which included *TMPRSS2* and *AR*, are important for PCa development regardless of race. In comparing AA versus EA PCa, 1876 unique genes undergoing dAS were identified, including *RASGRP2*, *NF1*, and *BAK1*. Over 2200 unique genes underwent dAS in AA versus EA normal prostate tissue, suggesting a differential role for these genes in normal prostate homeostasis. We identified splicing events involving 644 genes, including *PIK3CD*, *ITGA4*, and *MET*, that were present in both AA PCa and AA normal tissue, but were absent in EA

Table 2 Examples of differential alternative splicing events in AA PCa, EA PCa, AA normal, and EA normal specimens

Patient groups	# of AS genes	Examples
AA PCa and EA PCa	158	*TMPRSS2, AR*
AA PCa vs. EA PCa	1876	*RASGRP2, NF1, BAK1*
AA normal vs. EA normal	2205	*MTOR,EGFR, BCL2L1*
AA PCa and AA normal	644	*PIK3CD, ITGA4, MET*
AA PCa vs. AA normal	1575	*FGFR3, TSC2*

AA African American, *EA* European American, *PCa* prostate cancer, *TMPRSS2*, transmembrane protease serine 2, *AR* androgen receptor *RASGRP2* RAS guanyl releasing protein 2, *NF1* neurofibromin 1, *BAK1* BCL2 antagonist/killer 1, *MTOR* mechanistic target of rapamycin kinase, *EGFR* endothelial growth factor receptor, *BCL2L1* B-cell lymphoma 2-like 1, *PIK3CD* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta, *ITGA4* integrin subunit alpha 4, *MET* MET proto-oncogene, receptor tyrosine kinase, *FGFR3* fibroblast growth factor receptor 3, *TSC2* tuberous sclerosis complex subunit 2

specimens. We also identified 1575 unique genes (e.g., *FGFR3* and *TSC2*) undergoing dAS in AA PCa versus AA normal, but not EA PCa versus EA normal. These last two comparisons identify two important groups of splicing events: splicing events involving 644 genes that are inherited based on African ancestry, and splicing events involving 1575 genes that occur de novo during PCa progression solely in AA men. Over 70% of dAS events identified in AA PCa versus EA PCa occur in pathways known to contribute to oncogenesis (e.g., cell growth, proliferation, cell survival, cell adhesion, and DNA repair), and the majority were in-frame exon skipping events. Further validation of a subset of genes identified as potential targets for dAS was performed in an additional cohort of 22–25 AA and 21–24 EA specimens. Ninety-one percent of genes chosen for validation via RT-PCR were confirmed. The exon array results also identified 886 differentially expressed genes in AA versus EA PCa (compared to 1876 dAS genes). These data suggest that dAS is playing a much greater role in AA PCa disparities than differential gene expression.

Of the dAS genes identified, we focused on *PIK3CD*. *PIK3CD* codes for the p110δ (or PIK3Cδ) catalytic domain of the class I PI3Ks that bind the p85 inhibitory subunit. Upon activation by a receptor tyrosine kinase, p110δ phosphorylates phosphatidylinositol 4,5-bisphosphate (PftdIns(4,5)P2), generating phosphatidylinositol 3,4,5-trisphosphate (PIP3) which recruits AKT1 to the cell membrane thus activating downstream signaling cascades involved in cell growth, survival, and proliferation. The delta subunit of p110 is highly expressed in leukocytes [130, 131]. Four *PIK3CD* splice variants were identified: *PIK3CD-L* includes all 24 exons; *PIK3CD-Si* is missing exon 8 (encoding a domain between the Ras-binding and C2 domains); *PIK3CD-Sii* is missing exon 20 (encoding part of catalytic domain) (Fig. 2); *PIK3CD-Siii* is missing exon 8 and 20; and *PIK3CD-Siv* has a large deletion that encodes the helical domain and part of catalytic domain.

We selected the *PIK3CD-Sii* variant (identified as *PIK3CD-S* from here on) for further characterization for two reasons. First, the *PIK3CD-Sii* variant encodes a protein isoform missing 56 amino acids (Fig. 3) of the catalytic domain which has an important role in p110δ activity. Second, analysis of 494 PCa patients from The Cancer Genome Atlas (TCGA) revealed significantly decreased disease-free survival in patients with high *PIK3CD-S*/*PIK3CD-L* expression ratios ($p = 0.0052$). Although this analysis was performed irrespective of race or tumor grade, these data provide evidence for the clinical relevance of PIK3Cδ-S in PCa.

We performed siRNA-mediated knockdown of the *PIK3CD-L* variant in an EA PCa cell line VCaP (which has little to no expression of *PIK3CD-S*) and knockdown of either the *PIK3CD-L* or *PIK3CD-S* variant in an AA PCa cell line MDA PCa 2b (which expresses both variants). Knockdown of *PIK3CD-L* in VCaP cells results in a significant decrease in invasion, proliferation, and phosphorylation of key downstream signaling proteins (i.e., AKT, mTOR, and S6). In the AA cell line, knockdown of *PIK3CD-L* enriches expression of *PIK3CD-S*, leading to increased proliferation, invasion, and phosphorylation of AKT, mTOR, and S6. Not surprisingly, we observe no significant effects on invasion, proliferation, nor phosphorylation of signaling proteins after "knockdown" of *PIK3CD-S* in the EA cell line. However, a significant decrease in invasion, proliferation, and phosphorylation was observed after knockdown of *PIK3CD-S* (thereby enriching for *PIK3CD-L*) in the AA cell line.

In order to determine the effect of both variants on drug resistance, we ectopically overexpressed either PIK3Cδ-L or PIK3Cδ-S in two EA cell lines, VCaP and PC-3, and treated cells with the SMI CAL-101. CAL-101 (idelalisib (Zydelig®)) targets p110δ and is approved for treatment of hematological malignancies such as chronic lymphocytic leukemia, follicular B-cell non-Hodgkin lymphoma, and small lymphocytic lymphoma. Treatment of PCa cells overexpressing PIK3Cδ-L with CAL-101 results in a significant decrease in proliferation and AKT and S6 phosphorylation. CAL-101 treatment of PIK3Cδ-S overexpressing cells results in no significant suppression of proliferation or AKT and

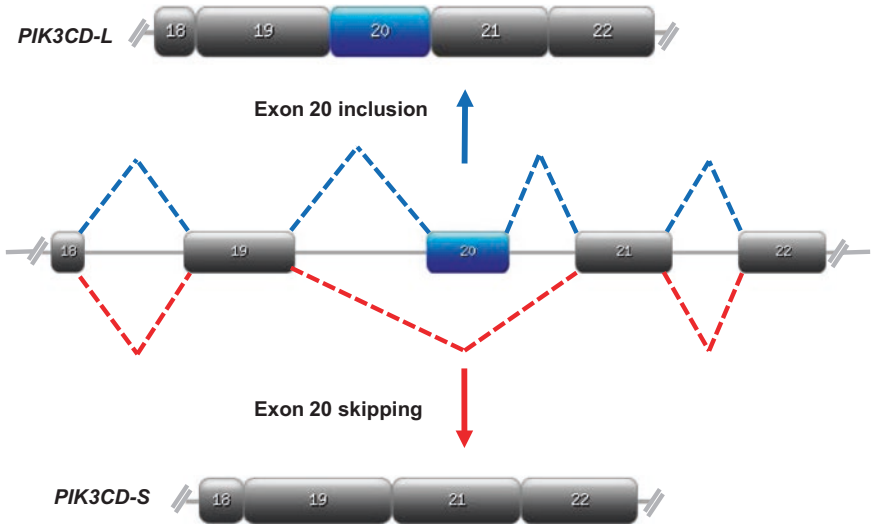

Fig. 2 Alternative splicing of *PIK3CD*. Schematic representations of alternative splicing of *PIK3CD* pre-mRNA. EA patients predominantly express *PIK3CD-L* which includes exon 20 (blue), while exon 20 is skipped (red) in AA patients to generate *PIK3CD-S*. Designed on https://prosite.expasy.org

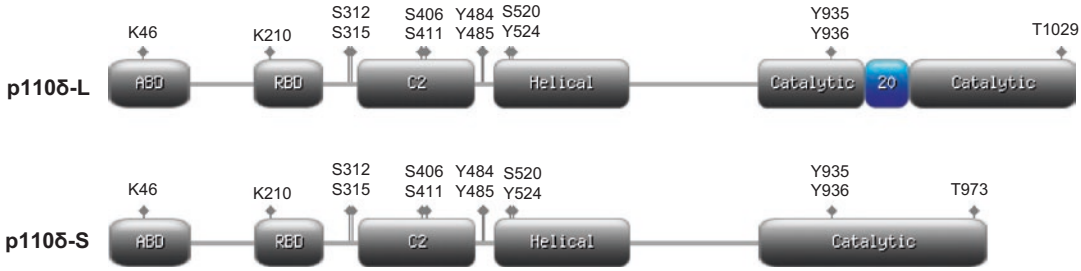

Fig. 3 Protein isoforms of p110δ due to alternative splicing. Schematic representations of long isoform due to exon 20 inclusion (top) and short isoform due to exon 20 skipping (bottom) of p110δ. Adaptor (p85) binding domain (ABD), RAS binding domain (RBD), C2, helical, and catalytic domains are shown in gray. Key phosphorylation and ubiquitin lysine (K), serine (S), tyrosine (Y), and threonine (T) sites are indicated as diamonds. The catalytic domain region encoded by exon 20 is shown in blue. Designed on https://prosite.expasy.org

S6 phosphorylation compared to vehicle-treated cells. In addition, PIK3Cδ-S expressing cells also have greater baseline proliferation compared to PIK3Cδ-L expressing cells.

Next, we investigated the effect of both PIK3Cδ isoforms on tumor formation, metastasis, and responsiveness to CAL-101 treatment in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. We observe significantly decreased tumor formation in mice injected subcutaneously with PIK3Cδ-L-expressing PCa cells and treated with 50 mg/kg CAL-101 compared to mice injected with PIK3Cδ-S-expressing cells and treated with CAL-101. Additionally, mice injected with PIK3Cδ-L-expressing cells via tail vein and treated with CAL-101 develop significantly less lung metastases compared to mice injected with PIK3Cδ-S-expressing cells and treated with CAL-101. These data suggest that CAL-101 is not effective against the PIK3Cδ-S isoform in vivo.

In order to test the functional differences between the two PIK3Cδ isoforms, we performed co-immunoprecipitation (co-IP) and cell-free kinase assays. Co-IP experiments demonstrate

that the PIK3Cδ-L isoform binds with a significantly higher affinity to the p85α regulatory subunit compared to PIK3Cδ-S. We also observe higher activity of PIK3Cδ-S in a cell-free kinase assay with and without the p85α subunit present and with or without wortmannin or CAL-101 treatment compared to PIK3Cδ-L. This suggests that PIK3Cδ-S activity is not as tightly suppressed by p85α and retains kinase activity even in the presence of SMIs such as CAL-101.

What has not been investigated up to this point is the mechanism responsible for the preferential expression of the *PIK3CD-S* variant in AA PCa. Therefore, we returned to gene expression data generated from previous studies [39, 132] to investigate which upstream SFs may be playing a role in the generation of the *PIK3CD-S* variant in AA PCa. Interestingly, we identified several SFs, including SRSF2, SRSF7, and HNRNPF, with increased expression in AA PCa compared to EA PCa at both the mRNA and protein levels (data not shown). Moreover, the intronic regions surrounding exon 20 of the *PIK3CD* pre-mRNA have computationally predicted binding sites for these three SFs. We hypothesized that binding of SRSF2, SRSF7, and/or hnRNPF to flanking regions of exon 20 in the *PIK3CD* pre-mRNA may facilitate exon 20 skipping, leading to the generation of *PIK3CD-S*. In order to test this hypothesis, we treated MDA PCa 2b cells with siRNAs targeting *SRSF2, SRSF7*, or *HNRNPF*, and observe a decrease in expression of *PIK3CD-S* and an enrichment of *PIK3CD-L* (Fig. 4a). We refer to this phenomenon as splice switching. SiRNA-mediated SF knockdowns are confirmed by western blot analysis (Fig. 4b). Our findings suggest that aberrant expression of SFs may be playing a role in the dAS observed in AA PCa.

Thus, we propose a mechanism in which "normal" expression of specific SFs (SRSF2, SRSF7, and/or hnRNPF) in EA PCa promotes inclusion

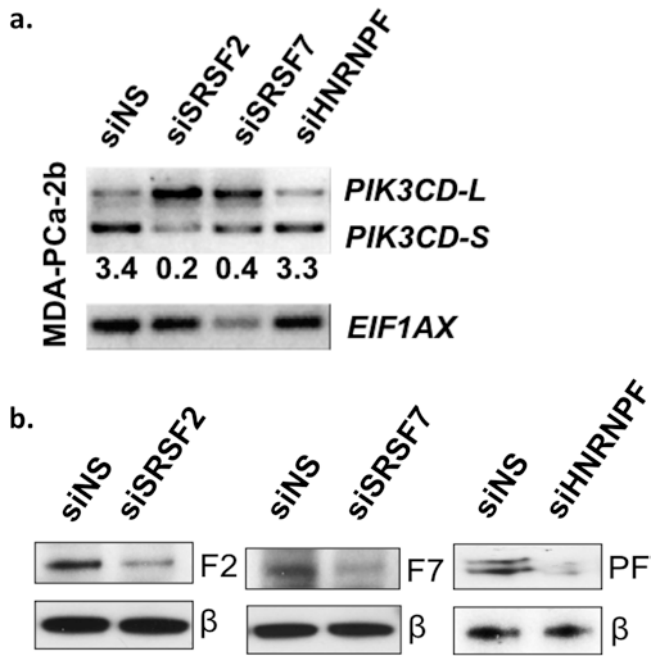

Fig. 4 Knockdown of overexpressed splicing factors causes splice switching of *PIK3CD* variants. (**a**) siRNA-mediated knockdown of three splicing factors in an AA cell line switches predominant expression of *PIK3CD* from the *−S* to the *−L* variant. Blots were quantified by densitometry and numbers underneath blots represent the *−S*/*−L* expression ratio. Shown are representative blots from 3–4 independent experiments. (**b**) Western blot confirms knockdown of splicing factors at the protein level. Abbreviations: F2, SRSF2; F7, SRSF7; PF, hnRNPF; β, β-actin. Shown are representative blots from 3 to 4 independent experiments

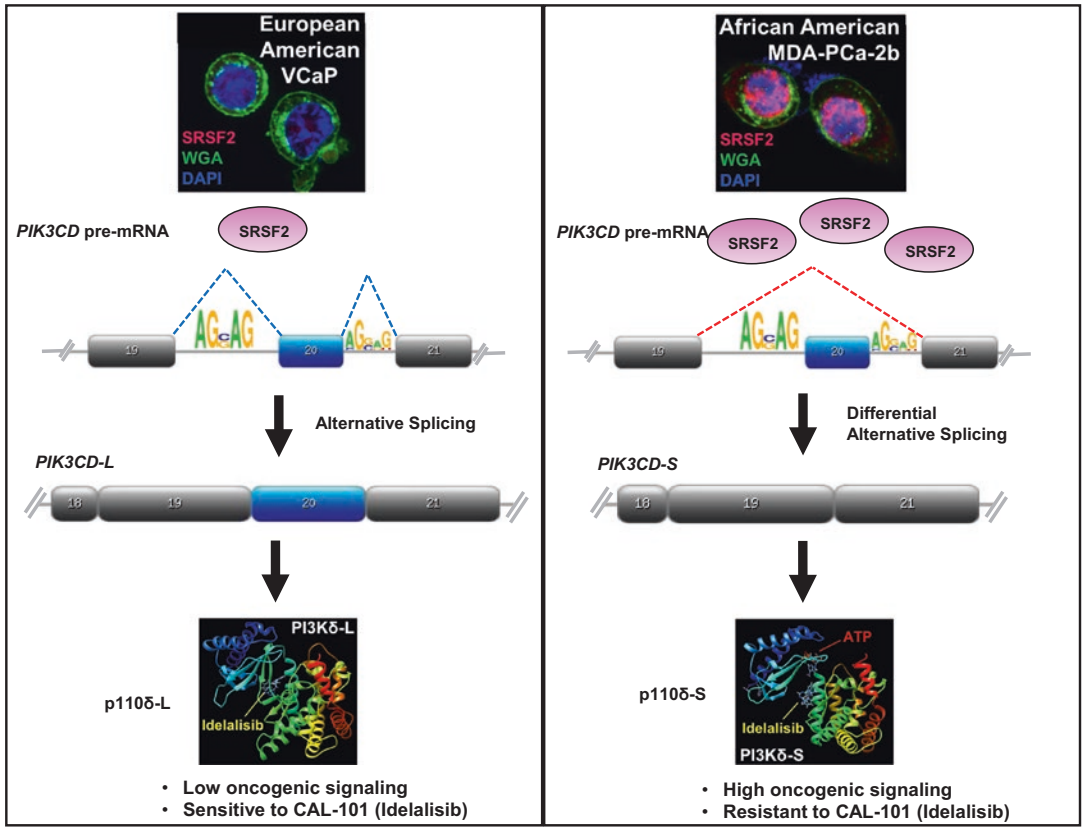

Fig. 5 Proposed mechanism for role of aberrant splicing of *PIK3CD* in PCa disparities. EA cell lines (e.g., VCaP) and patient specimens show "normal"/low expression of SFs, such as SRSF2 (left panel). Normal splicing of *PIK3CD* pre-mRNA generates the long variant containing exon 20, which encodes the p110δ-L protein that has low oncogenic properties. Aberrant over-expression of SRSF2 in AA cell lines (MDA PCa 2b) and patient samples results in differential alternative splicing of *PIK3CD* (right panel). This generates p110δ-S protein that has higher oncogenic signaling and is resistant to CAL-101 (idelalisib)

of exon 20 in the final transcript of *PIK3CD-L* and generation of the PIK3Cδ-L protein (Fig. 5). This protein isoform is sensitive to CAL-101 and has high binding affinity to the p85α regulatory subunit. In AA PCa, however, increased expression of SFs SRSF2, SRSF7, and/or hnRNPF results in dAS of the *PIK3CD* pre-mRNA, leading to skipping of exon 20, and subsequent generation of the PIK3Cδ-S protein isoform, which exhibits increased oncogenic signaling and decreased sensitivity to SMIs such as CAL-101 (idelalisib). Of interest, ~50% of patients treated second line with idelalisib for certain B-cell malignancies (e.g., chronic lymphocytic leukemia) will exhibit primary resistance to this SMI [133–135]. The mechanism of resistance is currently unknown. We propose that expression of the PIK3Cδ-S protein isoform may be responsible, in part, for this resistance. We are currently performing a high throughput chemical library screen to identify a SMI that will effectively suppress PIK3Cδ-S activity.

Conclusion

While studies focusing on PCa disparities have increased over the past 10 years, the RNA splicing landscape has not been fully characterized as a potential mechanism for race-related PCa aggressiveness. Our recent study has highlighted genome-wide dAS events occurring specifically

in AA PCa. The dAS events in AA PCa are over-represented in known oncogenic signaling pathways, possibly providing a mechanistic explanation for PCa disparities. While further studies are needed to fully understand the oncogenic capacity of other variants identified in our study (e.g., *FGFR3*, *MET*, and *TSC2*), these AS variants could serve as useful biomarkers for prognostic predictions and in identifying non-responsive patients to SMIs. Further characterization of dAS variants in AA PCa patients will provide greater, and much needed, insight into the mechanisms responsible for PCa disparities and possible new leads for therapeutic intervention.

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Discovery of Metabolic Biomarkers Predicting Radiation Therapy Late Effects in Prostate Cancer Patients

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Abstract

Patients presenting with prostate cancers undergo clinical staging evaluations to determine the extent of disease to guide therapeutic recommendations. Management options may include watchful waiting, surgery, or radiation therapy. Thus, initial risk stratification of prostate cancer patients is important for achieving optimal therapeutic results or cancer cure and preservation of quality of life. Predictive biomarkers for risks of complications or late effects of treatment are needed to inform clinical decisions for treatment selection. Here, we analyzed pre-treatment plasma metabolites in a cohort of prostate cancer patients (N = 99) treated with Stereotactic Body Radiation Therapy (SBRT) at Medstar-Georgetown University Hospital in a longitudinal, quality-of-life study to determine if individuals experiencing radiation toxicities can be identified by a molecular profile in plasma prior to treatment. We used a multiple reaction mass spectrometry-based molecular phenotyping of clinically annotated plasma

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samples in a retrospective outcome analysis to identify candidate biomarker panels correlating with adverse clinical outcomes following radiation therapy. We describe the discovery of candidate biomarkers, based on small molecule metabolite panels, showing high correlations (AUCs ≥ 95%) with radiation toxicities, suitable for validation studies in an expanded cohort of patients.

Keywords
Metabolomics · Prognostic biomarkers · Prostate cancer · SBRT

Introduction

Radiation therapy (RT) is an effective modality as a primary treatment of cancers, or as an adjuvant to surgery or chemotherapy. Risks, benefits, and late effects of radiation therapy are observed in the heterogeneous clinical responses of patients receiving curative radiation therapy. In principle, all cancers can be controlled if sufficient radiation doses can be delivered to tumors; however, in practice, the achievable radiation doses are frequently limited by toxicities that may result following exposure of normal tissues to high radiation doses [1]. Organ-specific tissue injuries following prostate irradiation may include acute toxicities (such as cystitis and proctitis), late toxicities (such as bleeding from the bladder or rectum), and broad toxicities (such as bone marrow depletion or soft tissue necrosis) [2–4]. Strategies to improve the therapeutic index of treatment with RT have focused on conformal radiation dose delivery technologies to target tumors more precisely, limit the volume of exposed normal tissues, and limit the doses delivered to normal tissues. These technologies include computer-assisted shaping of radiation doses, using intensity-modulated radiation therapy (IMRT), stereotactic radiosurgery (SRS), high-dose rate (HDR) brachytherapy, or particle therapies (e.g., proton beam therapy). Despite the sophistication of such technologies, the need to deliver high doses of radiation to tumors results in normal tissue toxicities in sensitive sub-sets of patients.

Variations in patients' normal tissue sensitivities to radiation have been attributed to genetic factors, including mutations in genes associated with DNA repair processes, immunological diseases, and connective tissue diseases. Extreme examples are provided by the genetic syndromes of ataxia-telangiectasia, Nijmegen breakage syndrome, and the clinical syndromes of scleroderma and systemic Lupus erythematosus [5]. Patients experiencing radiation therapy-related late effects experience symptoms many months after treatment. This underscores the need for clinical biomarkers to predict responses in a timely manner to support informing therapy decisions [6–8]. We tested the hypothesis that prostate cancer patients susceptible to adverse effects caused by radiation treatment-related toxicities carry a biochemical fingerprint that may be characterized using blood-based metabolite profiling. Furthermore, molecular changes may provide insight into specific pathway perturbations that further inform clinical therapeutic decisions. Based on this retrospective outcome study, we discovered candidate biomarker panels correlating with radiation responses. We recognize that these results will require validation in a larger, prospective clinical trial; however, we are also cognizant that this methodology may be applied to the validation study findings to improve the biomarker utility and to other modalities of treatment and other diseases.

Materials and Methods

Patient Recruitment and Study Population

Patients were enrolled at MedStar–Georgetown University Hospital into IRB protocol 2012-1175, an approved quality of life clinical trial. The protocol permitted longitudinal collection of clinical samples, symptom monitoring, and quality of life data, which have contributed to interim published reports of clinical outcomes including GU and GI acute and late effects [2, 9–15]. This study population is a part of ongoing recruitment of patients with prostate cancers coming in through the referral network to Medstar-GUH.

The study participants included Caucasians, African Americans, and men of other ethnicities, aged 35–70 years, residing in Washington DC and surrounding areas, who were diagnosed with localized prostate cancer by biopsy. Patients were recruited from the Departments of Radiation Medicine and Urology at the Medstar-GUH. All protocol enrolled participants completed informed consent for blood and urine collection and periodic self-reported symptom monitoring. Prior to enrollment, patients undergo physical examination, including a digital rectal examination (DRE). Phlebotomies (by trained phlebotomists) were performed prior to the first radiation treatment and at each subsequent following visit thereafter (1, 3, 6, 9, and 18 months after SBRT treatment).

Patient eligibility criteria included histologically confirmed adenocarcinoma of the prostate (biopsy within 1 year of enrollment); Gleason score 2–10; clinical stages T1c–T3c; no clinically or pathologically involved lymph nodes on imaging; no distant metastases on bone scan; measurement of prostate serum antigen (PSA) levels <60 days prior to registration; no history of pelvic radiotherapy, chemotherapy, or radical prostate surgery; no recent (within 5 years) or concurrent cancers other than non-melanoma skin cancers; no medical or psychiatric illnesses that would interfere with treatment or follow-up; a baseline AUA/IPSS score of <20; and no history of inflammatory bowel disease. All patients signed a study-specific consent form. Patients completed a detailed questionnaire regarding familial cancer history, tobacco use, medication use, occupational history, and socio-economic status, the 26-item EPIC (sexual, bowel, and urinary symptoms). Other patient data such as patient de-identifier number, prostatic volume, Gleason's grade, prior hormonal therapy, clinical co-morbidities, age, ethnicity, and body mass index were recorded. Blood samples were processed for serum and plasma collection. Buffy coat, mononuclear cells, and RBCs were collected and stored for future studies. All samples were processed within 4 h of collection, aliquoted and stored at −80 °C to preserve sample integrity. The clinical characteristics of the cohort in this study are detailed in Table 1.

Table 1 Clinical cohort characteristics

	Mean Age	Mean PSA	Mean Gleason
High risk group (n = 29)	71	24.50	8
Intermediate risk group (n = 50)	69	7.85	7
Low risk group (n = 20)	65	5.27	6

Prostate SBRT Treatment Planning and Delivery

Technical aspects of stereotactic body radiation therapy treatment planning and radiation delivery have been previously described [16, 17]. Briefly, ultrasound guided placement of gold fiducial markers is performed two or more weeks prior to thin-cut CT and high-resolution MRI imaging. The clinical target volume (CTV) includes the prostate and proximal seminal vesicles, to the bifurcation. The prescribed doses of 35–36.25 Gy are delivered in five fractions of 7–7.25 Gy over 2 weeks. Symptom management medications were prescribed based on the treating physician's clinical judgment, and urinary symptoms were managed with alpha-adrenergic antagonists and bothersome bowel symptoms were managed with anti-diarrheal medication (loperamide).

Mass Spectrometry-Based Molecular Profiling

We used stable isotope-labeled multiple reaction monitoring mass spectrometry (SID-MRM-MS) for quantitation of 350 metabolites. Metabolite extractions were performed using 25 μL of plasma sample, with 175 μL of 40% acetonitrile in 25% methanol and 35% water containing internal standards (stable isotope labeled). The samples were incubated on ice for 10 min and centrifuged at 14,000 rpm at 4 °C for 20 min. The supernatant was transferred to a fresh tube and used for UPLC-QQQ-MS analysis. Each plasma sample (2 μL) was injected onto a reverse-phase CSH C18 1.7 μM 2.1 × 100 mm column using an Acquity UPLC online with a triple quadrupole MS (Xevo TQ-S, Waters Corporation, USA) G2-QTOF system operating in the MRM mode.

Statistical Methods

After data pre-processing and ion annotation, the m/z values of the measured metabolites from plasma samples were normalized with log transformation that stabilizes the variance, followed by quantile normalization to make the empirical distribution of intensities the same across samples. Next, we performed feature selection using a ROC regularized learning technique, which uses the least absolute shrinkage and selection operator (LASSO) penalty. First, we obtained the regularization path over a grid of values for the tuning parameter lambda through tenfold cross-validation. Then, the optimal value of lambda, obtained by the cross-validation procedure, is used to fit the model. Finally, all the features with non-zero coefficients were retained as the candidate biomarker panel. This technique is known to reduce over-fitting and variance in classification.

The classification performance of the biomarker panel is assessed using the area under the ROC (receiver operating characteristic) curve (AUC). The ROC curve can be understood as a plot of the probability of classifying the positive samples correctly against the rate of incorrectly classifying true negative samples. Therefore, the AUC measure of a ROC plot is a measure of predictive accuracy. Due to the perfect separation for the classification, we also evaluated the panel using a robust method, the hidden logistic regression model with the maximum estimated likelihood (MEL) estimator. The resultant AUC scores turn out to be similar. We also calculated the AUC score for the regression with each marker, to rule out correlation with the patients' hormone therapy status.

Results

Metabolite Correlation with Adverse Outcomes for Biomarker Discovery

Analysis of metabolites offers to provide insight into molecular events occurring in a patient's cancer and in normal tissues. The patients in this cohort are uniquely positioned for the discovery phase study due to uniformity of treatment by a single radiation oncologist (SLC), standardized treatment planning and dosimetry and close follow-up management and publication of outcomes [18]. Reports of clinical outcomes have been comparable favorably to conventional radiation therapy and SBRT has been recognized as a standard of care option for treating prostate cancer patients.

Patients assigned into the conventional clinical risk groups of "low risk" (N = 20), "high risk" (N = 29), or "Intermediate risk" (N = 50) were included in this study. The risk categories are based on clinical evaluation strategies which account for PSA levels, Gleason's grade, age, and other co-morbidities. Although, a high percentage of the high-risk patients shows tumor recurrence, the clinical management of patients falling in the intermediate and low-risk group is based on "wait and watch" strategy. There are no anticipatory biomarkers that can help stratify a more susceptible sub-population in this group that may be at a higher than average risk for adverse outcomes of radiation therapy. We hypothesized that a specific plasma metabolic bio-signature, prior to radiation therapy, may characterize clinical susceptibility to late effects of radiation therapy. The overall study design is illustrated in Fig. 1.

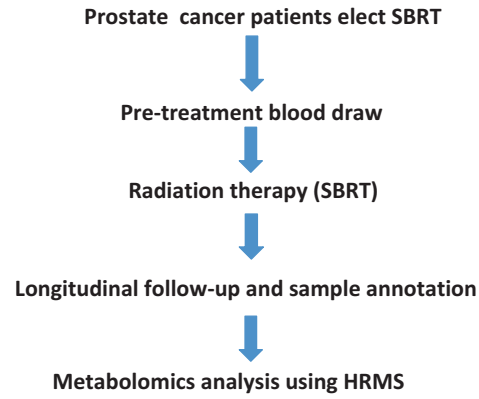


Fig. 1 Study design schema

Biomarker Panel Predictive of Rectal Proctitis

Our approach to identify pre-emptive markers of normal tissue toxicity due to radiotherapy was based on leveraging a state-of-the-art metabolomics analytical platform in conjunction with the clinical outcome data that were available for this cohort. Molecular profiles of pre-radiation samples from PC patients who developed rectal proctitis were compared to those who did not. Initially, we performed exploratory analyses to visualize overall differences in metabolic profiles of patients who developed radiation proctitis (within 8–12 months following SBRT), using PLS-DA (Fig. 2, Panel A) and interrogated the features contributing significantly to the group separation using volcano plots (Fig. 2, Panel B). We extended the same approach for developing predictive biomarker panels of adverse outcomes of radiation therapy. We performed retrospective outcomes analysis on a sub-set of patients who reported rectal toxicity/proctitis (N = 6) by comparing their pre-radiation plasma metabolite profiles with those of patients who did not experience rectal toxicity during the 2-year post-radiation monitoring period. A three-metabolite biomarker panel yielded an AUC of 97.8% (Fig. 3, Panel A). Using logistic regression, a plasma three-metabolite index was developed to stratify patients who later developed proctitis from those who did not develop symptoms during this interval, within a 95% confidence interval (Fig. 3, Panel B). The metabolite markers are listed in Fig. 3, Panel C. We found pantothenic acid and hypoxanthine to be upregulated while chenodeoxycholic acid/deoxycholic acid ratio (CDCA/DCA) to be downregulated in patients who developed radiation proctitis.

Biomarker Panel Predictive of Urinary Symptoms in Prostate Cancer Patients

Next, we asked if a comparison of pre-radiation profiles of patients who reported urinary late effects (flare) with patients who remained without such symptoms during this time would help develop a classification algorithm with high predictive accuracy. Flare usually occurs in a subset of patients about 1 year after SBRT and is characterized by lower urinary tract symptoms of increased urinary frequency, urgency, and a decreased stream [19]. Multivariate analysis using a PLS-DA model for plasma included two orthogonal components with R2 = 0.99 and Q2 = 0.62 providing support for the quality of the model (Fig. 4, Panel A), while the features contributing to the group differences (red dots) were visualized

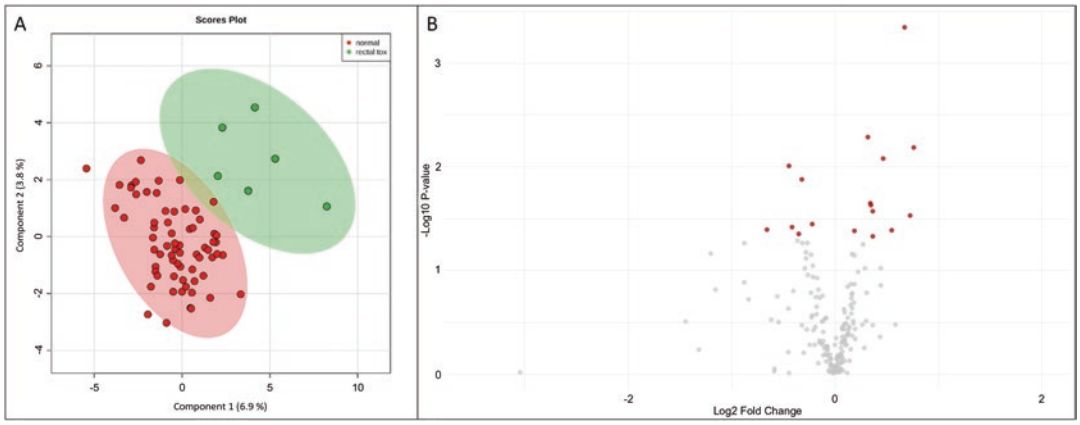
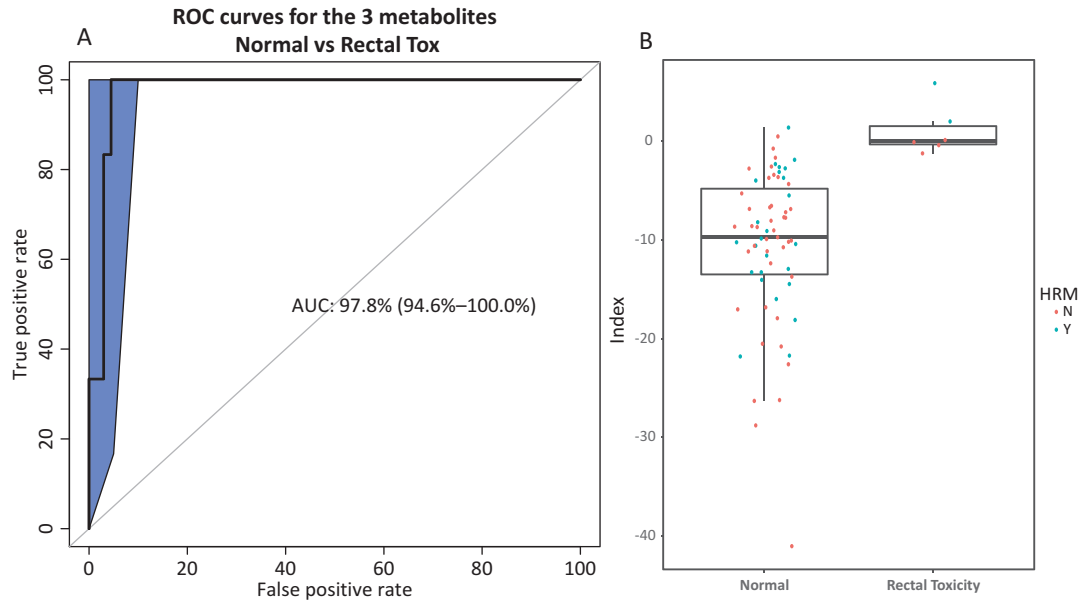


Fig. 2 Metabolomics yields a biomarker panel, predictive of radiation proctitis post-SBRT. **(a)** PLS-DA plot shows inter-group separation on the X-axis and intra-group heterogeneity on the Y-axis. **(b)** Volcano plot representing significantly dysregulated metabolites in the two comparative groups. Each red dot represents a metabolite with p-value<0.05 and fold change greater than 1.4 or less than 0.7



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Metabolites	Fold Change	p-value
Pantothenic Acid	↑	0.01
Hypoxanthine	↑	0.009
CDCA/DCA (bile acid)	↓	0.04

Fig. 3 Predictive biomarkers of proctitis post-prostate SBRT. (a) ROC curve for a 3 metabolite panel classifying patients experiencing proctitis. **(b)** Plasma Metabolite Index (PMI) plot demonstrates group stratification. The P3MI results based on the logistic regression model are illustrated as a boxplot that distinguishes between prostate cancer patients with and without proctitis post SBRT. Solid black horizontal lines represent the mean value, whiskers show the spread within a group. Orange and light blue dots represent PC patients receiving hormone therapy or not, respectively. **(c)** Biomarker panel (3 metabolites) predictive of proctitis

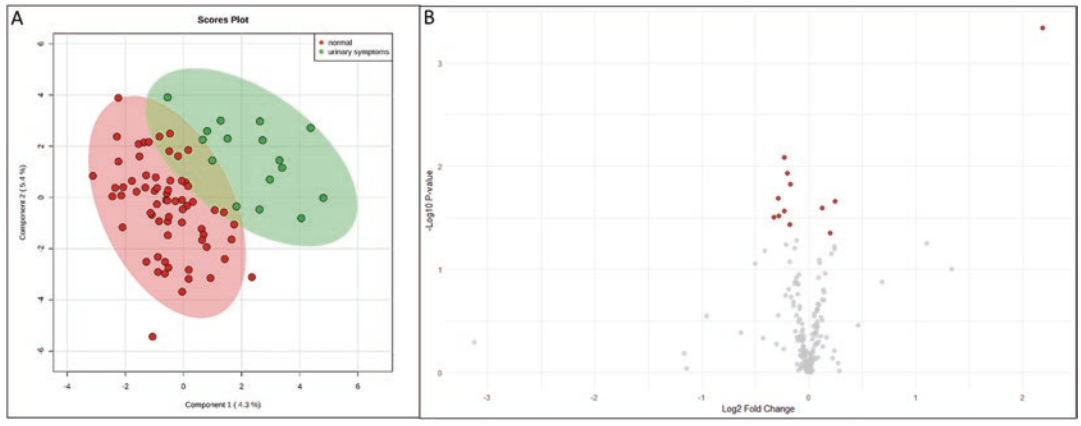
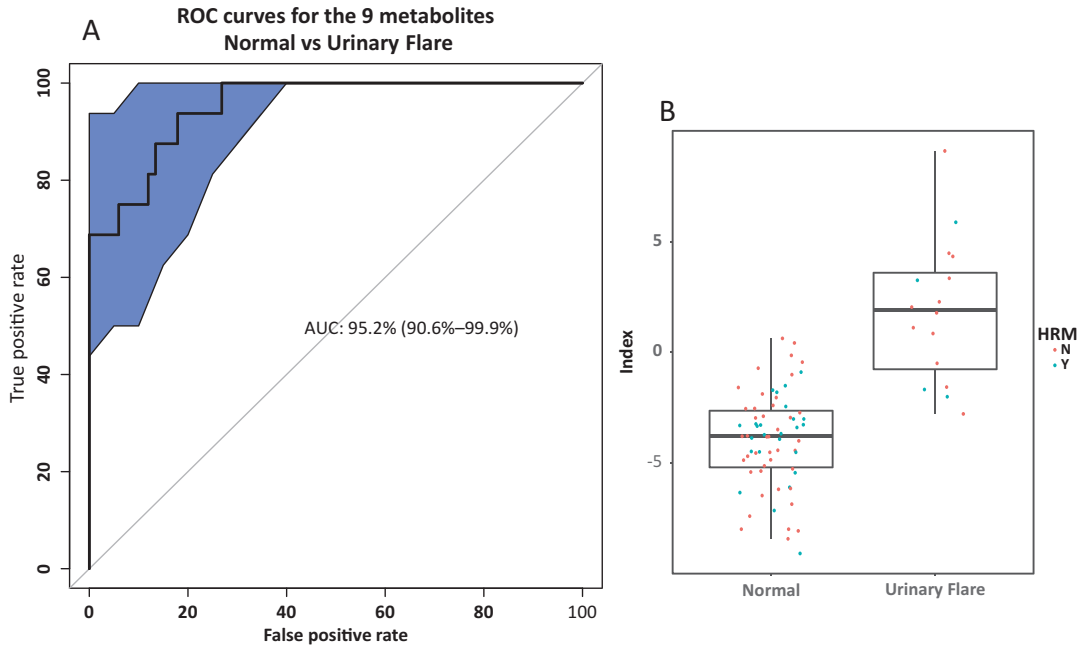


Fig. 4 Metabolomics profiling yields a biomarker panel, predictive of urinary flare post-prostate SBRT. **(a)** PLS-DA plot shows inter-group separation on the X-axis and intra-group heterogeneity on the Y-axis. **(b)** Volcano plot shows dysregulated metabolites in patients experiencing flare or not. Each red dot represents a metabolite with p-value<0.05 and fold change greater than 1.4 or less than 0.7



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Metabolite Name	Fold Change	p-value
C14:1-OH	↓	0.02
Pantothenic Acid	↑	0.004
Arginine	↓	0.03
Biotin	↑	0.01
LysoPC aa C14:0	↑	0.01
LysoPC aa C20:4	↑	0.01
PC aa C40:6	↓	0.02
PC aa C36:1	↓	0.009
PC ae C40:5	↓	0.02

Fig. 5 Predictive biomarkers of Urinary Flare post-prostate SBRT. **(a)** ROC curve for a 9 metabolite biomarker of urinary flare. **(b)** Plasma Metabolite Index (PMI) plot demonstrates group stratification. Solid black horizontal lines represent the mean value, while whiskers show the spread within a group. Orange and light blue dots represent patients receiving hormone therapy or not, respectively. **(c)** Table showing a 9 metabolites panel predictive of urinary flare patients treated with prostate SBRT

as volcano plots (Fig. 4, Panel B). Using LASSO (see methods), we were able to generate a nine-metabolite panel with an AUC of >95% (Fig. 5, Panel A). The plasma nine-metabolite index helped stratify the two comparative groups (Fig. 5, Panel B), while the metabolites comprising this panel are listed in Panel C. Several glycerophospholipids including PC aa C40:6, PC aa C36:1, and PC ae C42:5 were downregulated, while Lyso PC C14:0 and Lyso PC C20:4 were upregulated in patients experiencing urinary flare symptoms. An overall increase in Lyso PC/PC ratio suggests

an increase in systemic oxidative stress and inflammation in part due to increased phospholipase 2 activity [20]. Serum levels of biotin and pantothenic acid were also elevated in this subset of patients; interestingly the elevation of pantothenic acid has been implicated in breast cancer progression [21].

Discussion

Prostate cancer treatment has reached a technical plateau in terms of capability for dose escalation. Using modern conformal technology, a maximum tolerable radiation dose may be achieved in patients. Further escalations are undesirable due to a steep increase in the complication rate. The genetics underlying radiation sensitivities of patients are yet to be fully defined; however, omics approaches may offer an intermediate endpoint for risk stratification [22]. We provide metabolite panels as candidate predictive biomarkers for rectal and urinary late effects of radiation therapy. However, about 10% of patients treated for cancer experience radiation treatment-related late effects that adversely affect the quality of life [23, 24]. The manifestation of these symptoms takes months to develop and raises an urgent need for developing smarter strategies for symptom anticipation and management.

Currently there is no blood test that can be used to predict or monitor adverse symptoms in sensitive sub-populations of patients receiving radiation therapy. The value of using high throughput technologies, such as metabolomics, lies in the ability to develop predictive biomarker panels that can be used for identifying patients who are less likely to benefit from therapy because they are at risk of developing adverse symptoms. The underlying idea is to use this information to instruct personalized clinical therapeutics.

The objective of this study was to employ a high throughput metabolomics approach for delineating biomarker panels predictive of radiation-induced adverse effects in patients treated for prostate cancer. Such biomarkers would inform clinicians of risks for tissue toxicities in cancer patients and allow early intervention in patients at risk. We developed metabolite signatures predictive of recurrence and adverse responses to radiation therapy in a cohort of patients undergoing stereotactic body radiation therapy (SBRT) for prostate cancer. Sub-sets of patients developed rectal and urinary toxicities including symptomatic urinary flare (USF), and obstructed voiding symptoms/retention (UR) and radiation proctitis (RP). We hypothesized that individuals who are sensitive to radiation and develop toxicities carry a biochemical fingerprint that may be identified in the plasma profile.

By analyzing pre-radiation plasma samples, we were able to develop high accuracy predictive algorithms for urinary (nine-metabolite panel with AUC > 95%) and rectal toxicities (three-metabolite panel with AUC > 98%) in this cohort of prostate cancer patients. Interestingly, pantothenic acid was a common biomarker that appeared in both of the panels predictive of adverse symptoms in our prostate cancer cohort. Increased pantothenic acid (vitamin B5) has been thought to promote a glycolytic phenotype that is a hallmark of cancer cell proliferation. We also observed an increase in biotin. In general, Vitamin B1 metabolism has been linked to the typical anabolic metabolism for accumulating biomass essential to support proliferating cancer cells [25]. Our findings thus also implicate interesting biochemistry that can be leveraged as a metabolic vulnerability for better management of sensitive sub-sets of patients.

We also found changes in glycerophosphocholine (PC) metabolism, specifically an increase in overall serum Lyso PC levels with a concomitant decrease in PCs suggesting systemic and chronic inflammation and oxidative stress in patients who were susceptible to late effects. Lysophosphatidylcholines (lyso-PC) are products of phosphatidylcholine hydrolysis by phospholipase A2 (PLA2) and are present in cell membranes. Oxidized lipoproteins that result in the release of arachidonic acid and phosphocholines may lead to chronic stress-induced normal tissue toxicity. Patients who experienced tumor recurrence had lower serum levels of lithocholic acid, a metabolite that has been shown to have

anti-tumor effects in cultured human cancer cells by activating certain anti-cancer processes [26]. In the same set of patients, we also observed decreased levels of carnitine that is reportedly associated with increased pain sensation in patients with cancer [27]. Understanding the metabolic changes in cancer patients that are sensitive to adverse reaction of radiation therapy may be a key to devising better patient management strategies.

From a clinical diagnostic standpoint, development of these biomarker panels helps underscore the feasibility of a high throughput approach for predicting late effects of radiation therapy and lays the foundation for the development of strategies by which toxicity may be detected at an early stage and mitigated with intervention therapies. Although the candidate biomarker panels were developed in a defined cohort of patients undergoing prostate SBRT, we propose that the use of pre-treatment samples to identify the metabolite correlations may apply to other radiation modalities, such as IMRT and proton therapy, offering a broader context for these findings. One of the constraints of this study was the small sample size which did not allow us to perform an independent validation study for these biomarker panels. We propose that future validation studies include all RT modalities with larger clinical cohorts for prostate cancer treatment. We envision that such a biomarker panel may be useful for screening patients at risk of developing adverse symptoms subsequent to radiation therapy, in conjunction with clinical evaluation methodologies to improve strategies for patient management.

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Disclosures Anatoly Dritschilo, MD, owns equity and serves as CEO of Shuttle Pharmaceuticals, Inc.

Sean Collins, MD, PhD, receives grant support from Accuray, Inc.

Georgetown University has filed a patent application for the intellectual property associated with the discovered metabolite predictive biomarker panels.

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Part III

Skeletal Dysregulation and Cancer Spread to the Skeleton

The Aging Skeleton

David Goltzman

Abstract

Skeletal aging begins after peak bone mass is reached; progressive bone loss then occurs. Peak bone mass may occur at different ages in different skeletal sites and varies between sexes. Accelerated loss of bone occurs in the perimenopausal period in women, whereas more gradual but progressive loss of bone occurs in aging men. Changes in bone quality as well as bone quantity occur during growth and subsequent aging. These include changes in bone microarchitecture which may differ between cortical and trabecular compartments and in different sites, and may impact on bone size and geometry. Changes in material properties of bone matrix may also occur with aging. Loss of bone quantity and altered bone quality with aging may weaken bones and culminate in osteoporosis with an increased risk of fractures. Both genetic and epigenetic mechanisms may predispose to osteoporosis. Cellular and molecular events underlie the alterations in bone quantity and quality. Osteoclastic bone resorption and osteoblastic bone formation, tightly regulated by hormones, growth factors, and cytokines, are organized in coordinated activities resulting in remodeling and modeling. Malignancies, and anti-neoplastic therapies, may impact on the cellular and molecular events in the aging skeleton and produce focal or diffuse skeletal lesions and fractures.

Keywords

Bone remodeling · Osteoclasts · Osteoblasts · Osteoporosis · Malignancy

Overall Composition of Bone

Bone can be classified as consisting of in two major compartments: cortical bone, comprising 75–80% of skeletal mass, and defining the shape of bone, and trabecular bone (also called cancellous bone), consisting of a network of connecting plate and rod-like structures inside the cortical shell, and comprising 20–25% of skeletal mass. At a microscopic level, bone is composed of cells, and a matrix which is 67% inorganic, largely consisting of calcium (39%) and phosphorus (17%) and deposited mainly as hydroxyapatite crystals. The organic component (33%) of the matrix consists of protein, which is largely type I collagen and some non-collagenous proteins, and of mucopolysaccharides. The precise structure and composition of bone may vary with age and disease process.

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Cellular Components of Bone

The major bone cells consist of members of the osteoclast and osteoblast lineage. Osteoclasts are derived from hematopoietic stem cells (HSC) in the HSC niche (the anatomical location in which they reside) in bone marrow or from HSC arriving via the capillary blood supply. HSC may be quiescent, undergo self-renewal or differentiate through myeloid progenitors (CFU-M) either to the monocyte/macrophage lineage or through pre-osteoclasts to inactive multinucleated osteoclasts to active multinucleated osteoclasts. Active osteoclasts line the bone cell surface and are adapted to release enzymes and acid to resorb bone. Osteoblasts, in contrast, are derived from mesenchymal stem cells, and line the HSC niche in bone marrow. Mature bone-forming osteoblasts on the surface of bone synthesize and release bone matrix proteins and minerals, and may subsequently become embedded in mineralized bone as osteocytes. Osteocytes may sense mechanical stimuli and, via a network of canaliculi, release factors which modulate osteoblast and osteoclast function.

Tumor cells invading bone via blood and/or lymphatics enter bone marrow and bind to HSCs (Fig. 1). Tumor cells expressing the chemokine receptor CXCR4 home to osteoblastic cells expressing the chemokine protein CXCL12 and colonize the HSC niche [1]. Tumor cells may remain dormant in the HSC niche or may produce a variety of factors which activate osteoblasts and osteoclasts and result in osteoclastic osteolysis. These factors include transcription factors such as GLI2, runt-related transcription factor 2 (RUNX2), and hypoxia-induced growth factor 1α (HIF1α) that promotes osteolysis, and factors such as prostanoids and cytokines that stimulate osteoclasts. Jagged1 (Jag1) expressed on tumor cells may also activate osteoclast differentiation by inducing Notch signaling in pre-osteoclasts. Furthermore, tumor cells may release parathyroid hormone-related protein (PTHrP) which can bind to receptors on osteoblasts in bone marrow, enhance release of the tumor necrosis factor (TNF)-related cytokine, receptor activator of nuclear factor kappa-B ligand (RANKL), and diminish release of the RANKL antagonist osteoprotegerin [2]. RANKL binding

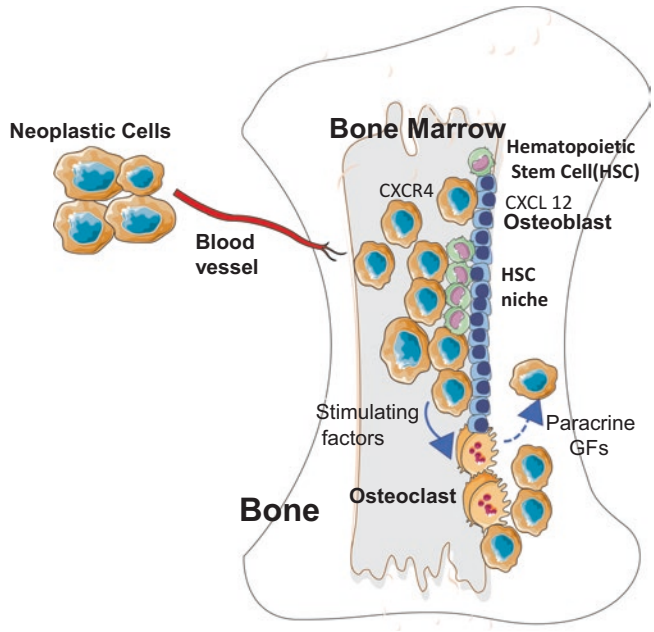

Fig. 1 Neoplastic cells travel to bone via blood vessels and/or lymphatics. CXCR4-expressing tumor cells home to CXCL 12-positive osteoblasts and colonize the hematopoietic stem cell (HSC) niche. Neoplastic cells are released from dormancy, and secrete osteoclast and osteoblast-stimulating factors. Osteoclastic bone resorption releases paracrine growth factors from the bone matrix which enhances neoplastic cell proliferation

to its receptor RANK on osteoclasts augments osteoclastic bone resorption, thus facilitating tumor cell penetration of bone. Osteoclastic osteolysis can release paracrine growth factors from the matrix which can then further enhance tumor cell proliferation.

Cellular Events in Skeletal Homeostasis

Modeling occurs when old bone is broken down at one site and new bone is formed in a different site within the same bone—the shape and position of bone may be changed in modeling, and modeling is thus a major process during growth. Modeling can still occur in adults, however, e.g., with aging, if excessive amounts of bone are removed from the inner (endosteal) surface, some new bone can be laid down on the outer (periosteal) surface, thus preserving the mechanical strength of the bone despite the loss of bone mass.

A second major cellular event in skeletal homeostasis is bone remodeling or turnover. In remodeling, a small amount of bone on the surface of trabeculae or in the interior of the cortex is removed and then replaced at the same site. Bone remodeling therefore occurs on the same surface of bone. This process is initiated when pre-osteoclasts are activated to become osteoclasts which then resorb a surface packet of bone. Pre-osteoblasts then migrate to the resorption lacuna that was produced, as the first event in the reversal process. Mature osteoblasts then differentiate and form new bone. The resorption defect is initially filled with new unmineralized bone or osteoid, which subsequently mineralizes to form mature bone. Active osteoblasts then become quiescent bone lining cells which cover the surface of bone. The complement of osteoclastic and osteoblastic cells that resorb an area of the bone surface and then fill it with new bone is termed the bone multicellular unit (BMU). Initiation of osteoclast activation in bone remodeling may occur in association with events such as calcium homeostasis, inflammation, bone microfracture repair, and mechanical loading. A genetic basis for bone remodeling and modeling is also present.

Osteoclast Regulation

Osteoclasts can be positively or negatively regulated by a number of humoral factors. Thus, osteoclasts may be very potently activated by RANKL, which is produced by osteoblasts and osteocytes, and by several other signaling molecules including macrophage-colony-stimulating factor (M-CSF), vascular endothelial growth factor (VEGF), and nitric oxide that are likely released from cells of the osteoblast lineage and endothelial cells [3]. A variety of cytokines released by immune cells, notably T cells in the bone marrow, including interleukin-1, interleukin-6, and tumor necrosis factor (TNF) alpha, can also stimulate osteoclasts. The egress of osteoclast precursors from the vasculature is stimulated by chemotactic factors including sphingosine-1-phosphate (S1P) via a process that is enhanced by 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] which, in high circulating concentrations, may also directly augment osteoclastogenesis. Osteoclasts can be inhibited directly by estrogens which prevent osteoclastogenesis and induce osteoclast apoptosis by acting on osteoclastic estrogen receptors α and β. Androgens appear to inhibit osteoclasts indirectly via conversion to estrogens by aromatization. Estrogens may also activate transforming growth factor (TGFbeta) which can inhibit osteoclast activity. Inhibins may also negatively regulate osteoclasts.

Osteoblast Regulation

Osteoclasts, by enhancing osteolysis, may facilitate release of a number of latent growth factors present in bone matrix, including insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), fibroblast growth factor (FGF), bone morphogenetic factors (BMPs), and TGF beta which can then activate osteoblasts. Direct communication between osteoclasts and osteoblasts by osteoclastic production of soluble osteoblast-stimulating factors termed clastokines has also been postulated; however, the role of these direct "coupling factors" have yet to be definitively

defined. A potent osteoblast-stimulating signaling system is the WNT growth factor system which can be inhibited by an osteocyte product called sclerostin. WNT1 is a B-cell product in bone marrow that promotes osteoblastic bone formation; however, other WNTs are also likely of importance in augmenting osteoblast activity. Mechanical loading and other stimuli that activate osteocytes may inhibit osteocytic release of sclerostin and thus allow enhanced WNT osteoblast-stimulating activity. Other circulating and locally released factors that can stimulate osteoblasts include PTH, PTHrP, androgens (by stimulating osteoblast precursors via androgen receptors), and osteblasts can be stimulated by a variety of circulating growth factors and cytokines.

Changes in Bone with Age

Loss of Bone Mass

Bone accrual increases during growth and development, reaches a peak bone mass (PBM), and then begins to decline as the organism ages. Reduced bone mass at old age, in one individual versus another, may result, in theory at least, if PBM that is achieved in one individual is lower than in another, or if the rate of bone loss with aging is accelerated in one individual relative to another individual even if the PBM achieved is the same in both. Both mechanisms may of course occur. Actual measurements of PBM, by determining bone mineral density (BMD) by dual X-ray absorptiometry (DXA), has shown that the age at which PBM is achieved may vary between regions of the skeleton and between males and females, e.g., PBM occurs earlier in the hip than in the spine in both sexes but is achieved earlier in males than females in the spine and earlier in females in the hip [4]. Similarly rates of bone loss after PBM occur differently in men and women. Thus in women, bone loss is markedly accelerated in the perimenopausal period and then continues at a lower rate, whereas in men, a persistent lower rate of bone loss occurs with aging [5].

There are genetic factors which clearly impinge on both PBM and bone mass loss with aging. Thus genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in the *WNT 16* gene and in the *ESR1/C6orf97* gene encoding the estrogen receptor alpha in premenopausal women with reduced bone mass, i.e., reduced PBM [6], whereas 56 loci (32 new) associated with low bone mass were identified in a very large GWAS done in postmenopausal women; several of these loci were associated with factors clustered within the pathways for mesenchymal stem cell differentiation, endochondral bone formation (e.g., PTHrP), osteoblast differentiation (Wnt signaling pathways), and osteoclast differentiation (RANK-RANKL-OPG system) [7].

Epigenetic mechanisms are critical regulators of the differentiation programs for cell fate and are subject to change during aging. Epigenetic regulation has also been associated with the development of osteoporosis associated with aging [8].

Loss of Bone Quality

Reduced bone strength (Fig. 2) can occur when bone quantity and/or bone quality is decreased, thus leading to increased fracture risk in the presence of a given mechanical load (e.g., a fall) and culminating in the systemic skeletal disease, osteoporosis. Bone quality is comprised of bone size, bone geometry, bone microarchitecture, and bone molecular architecture (material properties). Using an imaging technique termed high-resolution peripheral quantitative computed tomography (HR-pQCT), it has been possible to determine characteristics of bone microarchitecture in the radius and tibia. Thus, in younger individuals, cortical porosity due to increased osteoclastic resorbing activity in Haversian canals in cortical bone was lower in young women than in young men, but trabecular number and thickness were higher in young men than in young women. With aging, cortical porosity increased more in women than in men, trabecular thickness

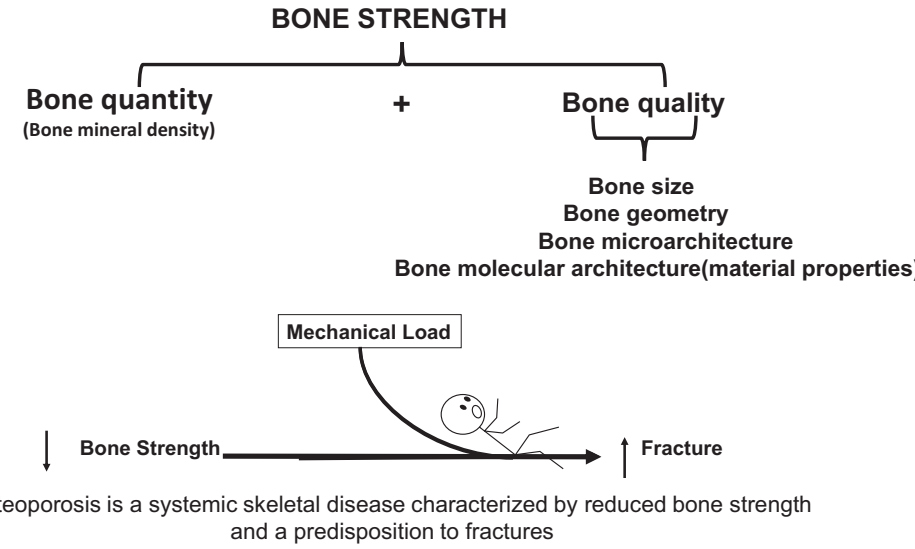

Fig. 2 Bone strength is comprised of bone quantity and bone quality. An estimate of bone quantity can be obtained by measuring bone mineral density (BMD) by dual X-ray absorptiometry (DXA). Bone quality comprises bone size, bone geometry, bone microarchitecture, and bone molecular architecture. With reduced bone strength, in the presence of a mechanical load such as a fall, there is an increased risk of fractures

declined more with age in men than in women, and expansion of bone at the outer (periosteal) surface increased more in men than in women so that total bone area was larger in men than in women [9]. By applying an engineering technique called finite element analysis (FEA) to HR-pQCT readings, it was possible to calculate mechanical strength of the bone and to determine that bone strength was 34–47% greater in young men than in young women and that the predicted change with age was similar in both sexes. The predicted increase in the mechanical load-to-strength ratio at the radius, an estimate of forearm fracture risk, was greater in women than in men. It was also possible to show that aging women with osteoporosis had thinner and more porous cortices than normal postmenopausal women [10]. It has also been reported that mineral to matrix ratios increase in trabecular bone with aging, and that carbonate to phosphate ratios and collagen cross-linking in the matrix increase with aging in both trabecular and cortical bones [11].

Modulation of Mechanisms of Skeletal Aging by Malignancies and by Anti-Neoplastic Therapies

Malignancies and Bone

A variety of malignancies notably those originating in breast and prostate may colonize bone, alter bone remodeling, and produce focal skeletal lesions. In addition, in the absence of metastases, malignancies may produce a variety of cytokines and growth factors, which can enhance bone turnover and produce diffuse demineralization of bone. Two notable such mediators are PTHrP and $1,25(OH)_2D$. PTHrP may act hormonally after release by a tumor which has not yet invaded bone, and may bind to its cognate receptor on osteoblasts, increase release of RANKL, and increase osteoclastic bone resorption thus causing reduced skeletal mass (osteopenia), and calcium release from bone which can lead to hypercalcemia [12]. Other tumors, notably lymphomas, may produce a highly active

25-hydroxyvitamin D 1alpha-hydroxylase enzyme, which can convert circulating levels of the inert precursor, 25hydroxyvitamin D [25OHD], to its active form $1,25(OH)_2D$ [13]. The increased active circulating $1,25(OH)_2D$ can enhance gut absorption of calcium; however, high circulating concentrations of $1,25(OH)_2D$ by its action on the vitamin D receptor (VDR) in osteoclasts can also increase osteoclastic bone resorption, cause generalized osteopenia, and liberate calcium from bone. Both mechanisms may thus contribute to the development of hypercalcemia. Both focal bone lesions induced by metastases and generalized osteopenia due to widespread increased bone resorption augment the risk of fractures over that already present in an aging skeleton.

Anti-Neoplastic Therapies and Bone

Anti-neoplastic therapies may also adversely affect the skeleton. Thus adjuvant therapy for breast cancer may induce a premature menopause and accelerate the normal processes of skeletal aging resulting in osteoporosis and increased fracture risk. Furthermore, hormone deprivation therapy for breast cancer and for prostate cancer may also have deleterious effects on the skeleton.

Major therapeutic approaches for estrogen receptor positive (ER+) breast cancer involve either blockade of estrogen production, blockade of estrogen action, or down-regulation of estrogen receptors [14, 15]. Blockade of estrogen production may involve ovarian ablation by oophorectomy, or in women with premenopausal ER+ breast cancer, administration of LHRH agonists (also called gonadotropin-releasing hormone or GnRH agonists) suppresses ovarian estrogen production ("medical castration"). Thus, LHRH agonists bind to pituitary receptors with greater affinity and a longer half-life than does endogenous LHRH, resulting in internalization of pituitary GnRH receptors, and causing the gonadotropic cells to become refractory to endogenous LHRH. Gonadotropin release is therefore inhibited, and ovarian estrogen production is reduced.

Reduced synthesis of estrogens from androgen precursors can also be achieved in postmenopausal women with ER+ breast cancer using aromatase inhibitors. In women, estrogens are critical for bone health and are derived by aromatization of adrenal and ovarian androgens, including dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), androstenedione, testosterone, and dihydrotestosterone (DHT), or by aromatization of peripheral androstenedione. Aromatase inhibitors may be reversible non-steroidal inhibitors, e.g., anastrozole and letrozole or irreversible steroidal inhibitors, e.g., exemestane. A number of adjuvant studies in breast cancer, using a variety of aromatase inhibitors, have shown increased fractures relative to treatment with the selective estrogen receptor modulator (SERM), tamoxifen [16].

Reversible blockade of estrogen action in ER+ breast cancer may be initiated with SERMs such as tamoxifen in contrast to inhibitors of estrogen production. Although SERMs are estrogen antagonists in breast, they may have weak estrogenic activity in bone and therefore may substantially spare the effects of estrogen loss in the postmenopausal skeleton. However bone loss may occur in premenopausal women because skeletal effects of SERMs are weaker than those of natural estrogens.

Finally, agents to down-regulate estrogen receptors, i.e., selective estrogen receptor down-regulators (SERDs) such as fulvestrant, may be employed which share the deleterious effects on the skeleton of blockade of estrogen production.

Androgen deprivation therapy (ADT) is used for castrate-sensitive non-metastatic prostate cancer and includes the use of orchiectomy, or LHRH agonists and antagonists, given either alone or in combination with androgen receptor antagonists. These agents can delay progression and prolong survival of prostate cancer but can also accelerate the development of osteoporosis. Thus, androstenedione is converted to estrone and testosterone to estradiol by aromatase activity in men as well as in women, and

estrogens are more important mediators than androgens of skeletal health in men as well as in women [17–19]. Consequently ADT, including both orchiectomy and GNRH agonists, via their action to reduce estrogen synthesis in men, can predispose to osteoporosis and increased osteoporotic fractures [20].

Currently anti-resorptive agents are available to prevent osteoporotic fractures in treatment-induced osteoporosis. This includes oral or intravenous bisphosphonates which appear to act by inhibiting osteoclastic farnesyl diphosphate synthase and osteoclastic aminoacyl tRNA synthetase, or denosumab which is a blocking antibody to the osteoclast-stimulating cytokine, RANKL [21].

Summary

Aging of the skeleton involves reductions in bone quantity and alterations in bone quality. Complex cellular and molecular mechanisms underlie these processes. Reductions in bone strength may lead to increased bone fragility and a predisposition to fractures. Neoplasms may impinge on the underlying skeletal mechanisms of aging to produce focal and generalized loss of bone and reduced bone strength. Anti-neoplastic adjuvant therapy may also impact negatively on the skeleton and accelerate aging. Pharmacologic therapy is available to retard the negative consequences of malignancy and its therapy on the aging skeleton.

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Conflicts of Interest None.

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Parathyroid Hormone-Related Protein (PTHrP): An Emerging Target in Cancer Progression and Metastasis

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Abstract

PTHrP was first discovered as the most common mediator of malignancy-associated hypercalcemia. Subsequently, the discovery of its ubiquitous expression in normal tissues unraveled its role as a physiological autocrine/paracrine regulator. The significance of PTHrP in cancer is not confined to malignancy-associated hypercalcemia, and sufficient evidence now also supports its role in skeletal metastasis through its modulation of bone turnover. Furthermore, our own studies have recently shown the critical role of PTHrP in breast cancer initiation, growth, and metastasis. More recently, we have provided new evidence that overexpression of PTHrP is associated with higher incidence of brain metastasis and decreased overall survival in triple-negative breast cancer patients. Further mechanistic studies in human and mouse model are necessary to fully understand the role of PTHrP in tumor progression and metastasis.

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PTHrP Background, Discovery, and Gene Structure

Background and Discovery

PTHrP was first identified after a nearly four-decade search of humoral factors that underlie the development of hypercalcemia in malignancy, a severe complication in patients with advanced-stage cancers. Fuller Albright, in 1941, was the first to postulate that ectopic production of parathyroid hormone (PTH), or a PTH-like factor, could be responsible for this "humoral hypercalcemia of malignancy" (HHM). He reported the case of a patient with renal carcinoma and a solitary bone metastasis associated with hypercalcemia and hypophosphatemia mimicking primary hyperparathyroidism [1]. PTH is an 84-amino acid hormone secreted exclusively by parathyroid cells, and it plays a key role in regulating Vitamin D, phosphate, and calcium homeostasis. Albright's hypothesis that cancer-secreted PTH was the putative etiological factor in HHM was a logical explanation due to its similarity to PTH actions. However, he was unable at that time to prove his hypothesis in the absence of specific immunoassay for the detection of PTH in the blood circulation of patients with HHM. Subsequently, several groups using PTH immunoassays determined that PTH blood levels in HHM were suppressed in response to calcium-induced inhibition of PTH by the parathyroid

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gland [2]. It was not until 1987, after great efforts were devoted to identifying and isolating this putative tumor-secreted factor, that three independent groups simultaneously identified a protein with similar biological activities and homology to the amino terminus of PTH, therefore named PTHrP. An active peptide with a molecular weight of 18-kDa was isolated by one group from a human lung cancer cell line, a second group from a 6-kDa active peptide from human renal carcinoma cells, and a third group from a 17-kDa active peptide from human breast carcinoma [3–5]. Subsequently, the complementary DNA (cDNA) encoding these peptides confirmed that eight of the initial 13 N-terminal amino acids of PTHrP were identical to those of human PTH [6–8]. These findings explained the biochemical similarities between primary hyperparathyroidism due to excessive production of PTH and HHM from cancer-producing PTHrP.

Gene Structure

The human PTHrP gene (*PTHLH*) is located on the short arm of chromosome 12, distinct from the human *PTH* gene, which is located on the short arm of chromosome 11. The similarities in overall genomic organization and similar exon–intron boundaries of *PTHLH* and *PTH* genes give compelling evidence of their origin from a common ancestral gene which evolved from a gene duplication event during evolution [9] (Fig. 1). The human *PTHLH* gene spans ~15 kb of genomic DNA and includes nine exons that are transcribed by three functionally distinct promoters (Fig. 1). The canonical TATA promoters (P1 and P3) are located upstream of the noncoding exon 1 and exon 4, respectively, whereas 5′ to noncoding exon 3 is a GC-rich promoter (P2) (Fig. 1). Although it is not completely understood whether PTHrP transcription is controlled by each of these promoters in a tissue-specific manner, all species identified to date share only one functional promoter equivalent to the human P3, but not P1 or P2 promoters. Therefore, it is likely that the dominant transcriptional regulation of PTHrP operates through the P3 promoter [10]. Gene sequence comparison reveals that exons equivalent to human exons 4, 5, 6, and 9 are highly conserved among species, which suggests that these exons may constitute the basic *PTHLH* gene structure with important biological functions (Fig. 1).

Alternative splicing gives rise to three separate human PTHrP isoforms which differ at their carboxyl-terminal ends and contain either 139, 141, or 173 amino acids. Although there is no intron between exons 6 and 7 in the human *PTHLH* gene, the junction provides a splice donor site for acceptors at the beginning of exons 8 and 9 (Fig. 1). Exon 6 encodes a region, common to all three isoforms, whereas exons 8 and 9 encode the exclusive carboxy termini of PTHrP 1-173 and 1-141 isoforms (Fig. 1). The mRNAs for each of these isoforms are commonly expressed in various normal and cancer tissues in humans, but the full characterization of their tissue distribution, processing, and function remains to be established. In contrast to the human *PTHLH* gene organization, the gene organization in other species is relatively simple, with a single promoter producing one single isoform. In rat and mice, mature peptides of 141 and 139 amino acids, respectively, are expressed [11, 12]. The *PTHLH* gene yields a single mature peptide of 139 amino acids in chicken and 126 amino acids in fugu [13, 14].

PTHrP Physiology

PTHrP Protein Structure, Functional Domains and Its Receptor

PTHrP amino acid sequences uncovered several functional domains including a prepro sequence, a PTH-like region, a nuclear localizing sequence (NLS), and a C-terminal domain named osteostatin (Fig. 2). The intracellular “prepro” precursors −36 to −1 of the mature peptide are necessary for intracellular trafficking and secretion of the PTHrP polypeptide [10]. The PTH-like region is essential for nearly all the agonist actions of PTHrP at the classical PTH/PTHrP type 1 receptor (PTH1R), a class II G-protein

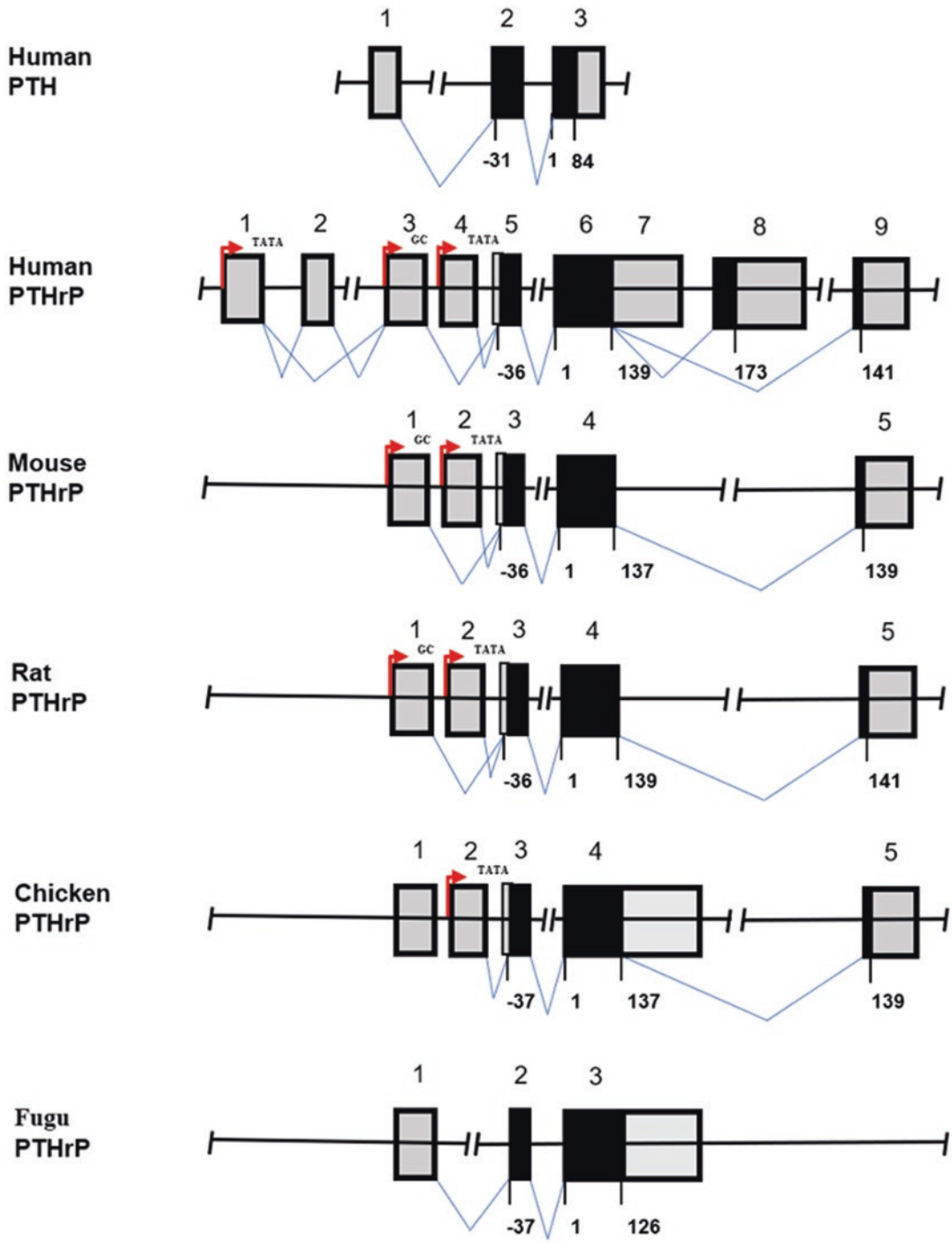

Fig. 1 Gene structures of human, mouse, rat, chicken, and fugu PTHLH with the human PTH genes. The coding regions and untranslated sequences are indicated by the black and gray boxes, respectively. Exons are noted with Arabic numerals. The positions of the three promoters (red arrow) of human PTHLH are shown (P1, TATA; P2, GC-rich; P3, TATA). The known splicing events in human PTHLH are shown

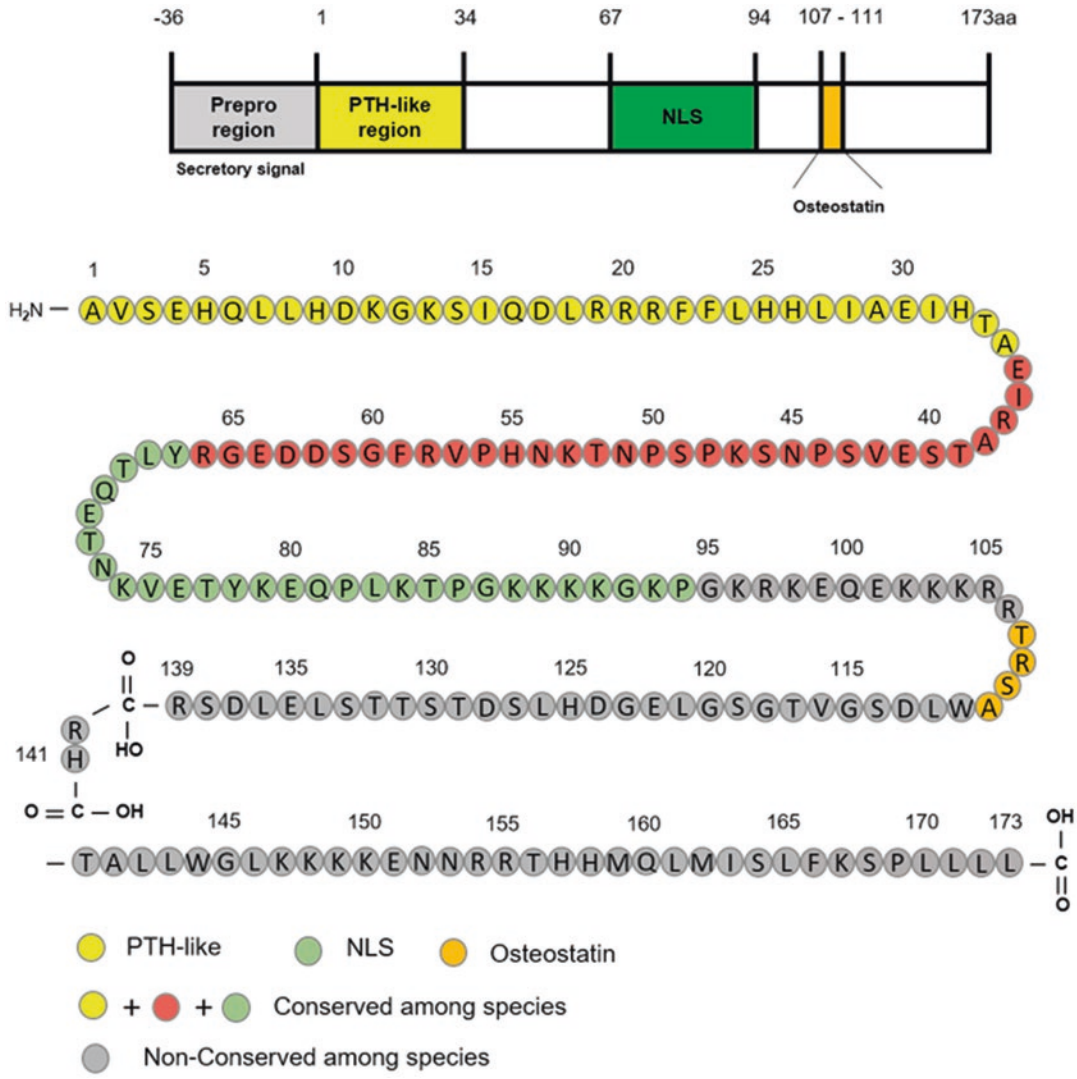

Fig. 2 Human PTHrP functional domains and amino acid sequences. The three isoforms resulting from alternative splicing have 139, 141, and 173 amino acids (aa), respectively. The prepro region (gray) includes the signal sequence (aa −36 to −1). The PTH-like region (yellow) binds to the PTH1R receptor (aa 1–34). The region responsible for nuclear localizing sequence (NLS) (green) is (aa 67–94). The osteostatin region (orange) is (aa 107–111)

coupled receptor, expressed on classic PTH target tissues (bone and kidney) that regulate calcium and phosphate homeostasis. This PTH-like region covers the first N-terminal domain of PTHrP (amino acids 1–13), which shares the highest degree of primary sequence homology with PTH (8 of the first 13 residues are identical). The following 14–36 region of PTHrP has no homology with the primary amino acid sequence of PTH; however, it is critical for the binding of PTHrP to the PTH1R. Competitive binding assays have shown that PTH (1–34) and PTHrP (1–36) bind PTH1R with almost equal affinity, whereas shorter N-terminal fragments of either PTH or PTHrP do not [15]. This indicates that the binding domain of PTHrP (14-36) has a similar secondary structure to PTH, regardless of the differences in primary structure.

Upon binding to the PTH1R in bone and kidney, PTHrP can activate intracellular cyclic 3′, 5′-adenosine monophosphate (cAMP) which further activates both the adenylyl

cyclase/protein kinase A (PKA) pathway as well as the calcium/inositol phosphate/protein kinase C pathway [16]. PTHrP is frequently coexpressed in the same cells or expressed in those immediately adjacent to PTH1R. Such close juxtaposition of cells expressing PTH1R and PTHrP highlights its function as a paracrine/autocrine factor in many tissues [10]. For example, PTHrP/PTH1R signaling system is crucial not only for physiological functions of PTHrP in bone and mammary gland development but also for pathological effects of PTHrP as a circulating, tumor-derived factor in HHM, as well as a locally produced factor at metastatic sites.

The mid-region (amino acids 37–106) of PTHrP includes a nuclear localization sequence (NLS) within residuals 67–94, which can translocate cytoplasmic PTHrP into the nucleus. The NLS of PTHrP contains a basic amino acid sequence, so called cell-penetrating peptides (CPPs), which is homologous to the NLS found in human retroviral regulatory protein [17]. The putative length of NLS was defined by Lam and colleagues to reside between residues 67–94 [18]. The PTHrP import mechanism involves the NLS forming a complex composed of importin β and the monomeric GTP-binding protein Ran which is then transported into the nucleus through the nuclear port complex [10]. PTHrP residues 83–93 are essential for importin β recognition, with residues 71–82 required for high-affinity binding [19]. The crystal structure of a fragment of importin β bound to the non-classical NLS of PTHrP provides the most important molecular evidence for supporting this region as the NLS [20]. This is a strong evidence to support the intracrine role of PTHrP aside from its autocrine/paracrine actions.

Although the actions of PTHrP in the nucleus are not fully understood, current evidence indicates that it plays important roles in both normal and malignant cells. PTHrP can be targeted to the nucleus in vascular smooth muscle cells and this nuclear targeting is associated with an increase of vascular smooth muscle cell proliferation [21]. In breast, colon, and prostate cancer cells, the nuclear pathway was found to stimulate cell proliferation, prevent tumors cells from apoptosis or anoikis, and stimulate cell migration [22].

Finally, the C-terminal region consisting of amino acids 107–139 is associated with inhibition of osteoclast function and stimulation of osteoblast proliferation [23–25]. It has also been found that this region together with the NLS can increase mitogenesis in vascular smooth muscle cells [26]. However, it should be noted that this C-terminal region is the least conserved domain among all species, the human and mouse having only seven residues, and rat and human having 13 residues in common. The functions of the C-terminal region of PTHP remain controversial and need further investigation.

Normal Physiological Functions of PTHrP

PTHrP is expressed in many normal cell types and tissues and therefore normal physiological functions have been proposed. In normal physiological condition, PTHrP acts as an endocrine factor only in two identified circumstances: (1) in lactation where PTHrP is produced by the breast and reaches the circulation [27]; (2) in the fetus, where it regulates maternal fetal calcium transport [28]. In most cases, PTHrP is regarded as having an autocrine or paracrine role in normal development and postnatal physiology. Here, we will focus on the functions of PTHrP in bone and mammary gland development in order to better examine its pathological role in breast tumor initiation, progression, and bone metastasis.

PTHrP in Mammary Gland Development

A human monogenetic disorder and animal models have shed light on the crucial role of PTHrP in mammary gland development [29]. Fetuses with Blomstrand chondrodysplasia lack breast tissue, indicating that PTHrP is essential for breast development in humans [30]. Studies in *Pthlh*- and *Pth1r*-null mice show that PTHrP signaling is indispensable for the formation of mammary glands [29]. The mammary gland formation in embryos is regulated by a cross-talk between the epithelial cells in the bud and ducts and adjacent

mesenchymal cells in the stroma [29]. In mice and human fetuses, PTHrP is expressed by the epithelial cells in the mammary bud, which interacts with the PTH1R expressed by the surrounding mesenchyme [31]. PTHrP–PTH1R cross-talk is required for the differentiation of the mesenchymal cells, which in turn stimulates the outgrowth of the epithelial ducts [31]. Disruption of either PTHrP or PTH1R interrupts the cross-talk between epithelium and mesenchyme, leading to a failure of mammary development in mice and humans [31]. Potential downstream signaling regulated by PTHrP includes Wnt and bone morphogenetic protein 4 (BMP4) as well as upregulation of several transcription factors including muscle segment homeobox 2 (Msx2), androgen receptor, and lymphoid enhancer-binding factor 1 (Lef1) [32, 33]. After embryogenesis, PTHrP is highly expressed by alveolar epithelial cells during lactation and is secreted into milk and into the circulation [27].

PTHrP in Bone Development

Generation of mice homozygous for a disrupted *Pthlh* gene established the first direct evidence for a critical role for PTHrP in the process of normal skeletal development. *Pthlh*-null mice die at birth, probably of asphyxia, and exhibit widespread abnormalities of endochondral bone development [34]. Subsequent studies characterized this phenotype of chondrodysplasia as a consequence of diminished proliferation, accelerated differentiation, and premature apoptotic death of chondrocytes [16]. Jansen's metaphyseal chondrodysplasia (JMC), a rare autosomal dominant human disorder, provided the first evidence that PTHrP actions in endochondral ossification are mediated by PTH1R [35]. Four different mutations in the *PTH1R* gene have been described in patients with JMC which lead to a constitutive, PTHrP-independent receptor activation [35]. Such activating *PTH1R* mutations in humans with JMC and overexpression of the same constitutively active receptor in the growth plate of transgenic mice gave rise to a delayed endochondral bone formation phenotype [35, 36]. Overexpression of Pthrp in chondrocyte of transgenic mice results in a similar pattern as observed in mice with active Pth1r overexpression in chondrocyte [37]. Alternatively, mice with homozygous *Pth1r* disruption exhibit accelerated differentiation of chondrocytes, and a more severe but similar phenotype to the one observed in *Pthlh*-null mice [38]. Together, loss-of-function mutations in *PTH1R* gene causes skeletal abnormalities observed in infants with Blomstrand chondrodysplasia, which are mirror images to those observed with JMC [39]. These findings provide sufficient proof that PTH1R mediates most of the cartilaginous effects of PTHrP.

It is interesting to note that the phenotype of *Pth1r*-null mice does not fully recapitulate the one observed in *Pthlh*-null mice, indicating that PTHrP may exert additional effects in a PTH1R-independent fashion. Knock-in mice homozygous for a truncated Pthrp 1–84, missing the NLS and the C-terminal region but preserving their ability to bind with Pth1r were generated [40]. These knock-in mice displayed retarded growth, early senescence, and malnutrition leading to their rapid postnatal demise [40]. This model established a pivotal role for nuclear PTHrP in promoting cellular proliferation while inhibiting pathways leading to senescence.

Pthlh-null mice not only unraveled the central role of PTHrP in endochondral bone formation but also revealed the importance of PTHrP postnatally in bone remodeling. Bone is a dynamic organ that undergoes continuous remodeling. Bone homeostasis depends on the balanced activities between osteoblasts (mesenchymal stem cell-derived bone-forming cells) and bone-resorbing cells of monocyte and macrophage lineage known as osteoclasts (Fig. 3). First, in the *Pthlh*-null mice, osteoblastic progenitor cells contain an inappropriate accumulation of glycogen, which indicates a metabolic defect in cells of the osteogenic lineage secondary to PTHrP deficiency [41]. Second, heterozygous *Pthlh*-null mice are born phenotypically normal, but exhibit a low bone mass by 3 months of age characterized by a marked decreased in trabecular thickness

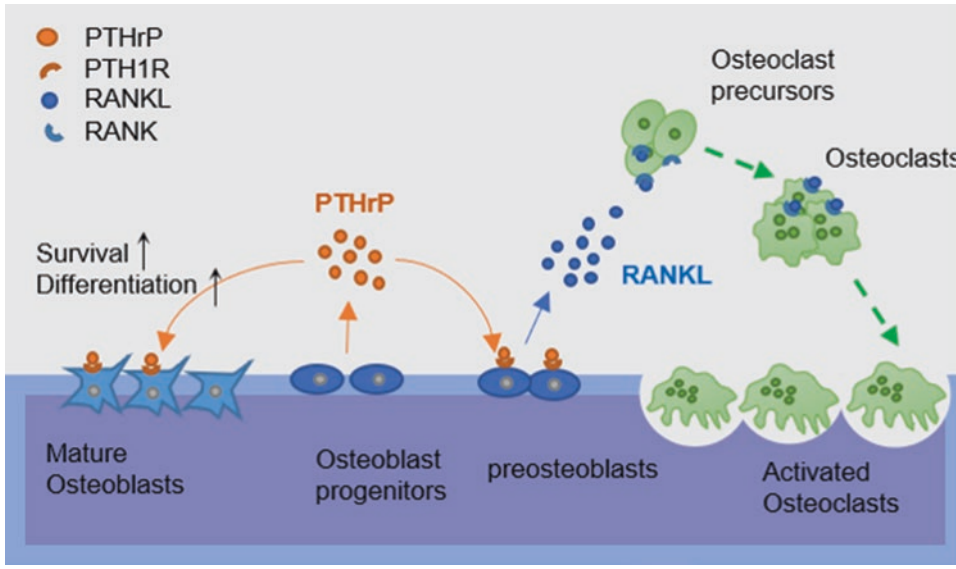

Fig. 3 Paracrine actions of PTHrP in bone remodeling. PTHrP produced by cells of the early osteoblast lineage acts on more advanced differentiated cells possessing the PTH1R, therefore promoting their differentiation and bone formation and while inhibiting apoptosis of mature osteoblasts. Moreover, PTHrP increases production of RANKL and binding of RANKL to RANK on hematopoietic progenitors leading to enhanced differentiation of osteoclast precursors and osteoclasts activation

and connectivity, and an abnormally high number of adipocytes in the bone marrow [42]. Third, PTHrP and PTH1R are expressed at different stages of the osteogenic lineage, indicating its pivotal role in the regulation of the maturation of pluripotential bone marrow stromal cells toward the osteogenic lineage [16].

During times of bone homeostasis, osteoclast activity and maturation is regulated by the interaction between its receptor activator of nuclear factor κB (RANK) surface receptor and that of the receptor activator of nuclear factor κB ligand (RANKL) protein expressed by osteoblasts and other bone marrow stromal cells; whereas osteoprotegerin (OPG), a decoy receptor for RANKL, diverts RANKL binding to RANK, reduces the half-life of membranous RANKL, and therefore inhibits bone resorption induced by osteoclasts [43]. PTHrP can stimulate bone resorption indirectly by upregulating the expression of RANKL in stromal osteoprogenitors [44] (Fig. 3). Binding of RANKL to RANK, a membrane protein expressed by hematopoietic progenitors, results in increased differentiation of osteoclast precursors and maturation (Fig. 3).

PTHrP and Cancer Biology

PTHrP and Malignancy-Associated Hypercalcemia (MAH)

MAH is a well-known complication of cancer and occurs in about 20–30% of cancer patients [45]. MAH is classified into four groups: humoral hypercalcemia of malignancy (HHM), local osteolytic hypercalcemia (LOH), excess $1,25(OH)_2D$ production, and ectopic PTH secretion. In 1936, Gutman et al. identified LOH in patients suffering from multiple myeloma and breast cancer (BC) with extensive bone lesions [45]. In 1941, Fuller Albright proposed a mechanism of hypercalcemia independent of bone metastasis [1]. The existence of a systematic factor with PTH bioactivity was suggested when Albright described a patient with renal carcinoma, hypercalcemia, and hypophosphatemia following an irradiation of bone [1]. At present, hypercalcemia secondary to PTH-like mediators accounts for approximately 80% of the cases, whereas LOH accounts for most of the remaining cases in cancer patients [45]. Excess $1,25(OH)_2D$

production accounts for <1% of all cases and ectopic PTH secretion by tumor cells is an even rarer event [45].

The classic signs and symptoms of severe hypercalcemia include confusion, constipation, nausea, anorexia, and coma. Elevated PTHrP in the context of hypercalcemia is almost always associated with PTH suppression [45]. A low or low-normal serum phosphorus level, if present, confirms the diagnosis [45]. There is still some controversy about the $1,25(OH)_2D$ circulating level in HHM which has been found to be either suppressed, normal, or even elevated [46–48]. Breast, renal, and squamous carcinomas are the most common solid cancers associated with hypercalcemia [45]. Among hematological malignancies, multiple myeloma is the most commonly associated with hypercalcemia, followed by leukemia and non-Hodgkin's lymphoma [45]. Circulating PTHrP levels have been reported to be elevated in 50–90% of hypercalcemic cancer patients with solid tumors and in 25–60% of patients with hematological malignancies [49]. In addition, circulating PTHrP may also have prognostic value. A prospective study conducted by our team in patients with MAH indicated that elevated circulating PTHrP is an indicator of poor prognosis and is associated with reduced survival (Fig. 4) [51]. Several other studies also confirmed the prognostic value of PTHrP [52, 53].

However, measurement of serum PTHrP presents few challenges. First, tumors may express several isoforms of PTHrP which could either circulate intact or be subjected to further metabolism. The nature of these potential circulating forms is still elusive. Second, it has been challenging to establish appropriate immunoassays that can recognize these multiple circulating either bioactive or inactive forms. Shortly after the discovery and characterization of PTHrP, immunoassays were developed which focused on the recognition of both the N-terminal (1–36) bioactive region and the inactive C-terminal (109–136) region of PTHrP [54]. However, little or no immunoreactivity was detected in the circulation of patients with MAH indicating that the three intact isoforms were likely metabolized. Burtis et al. also designed a two-site immunoassay using two polyclonal antibodies, a capture antibody raised against PTHrP 37–74, and a radiolabelled signal antibody raised against PTHrP 1–36 [54]. Most patients with HHM had elevated PTHrP levels indicating that PTHrP fragments containing at least the first 74 amino acids were present in a variety of hypercalcemic cancer conditions [54]. In addition, Burtis et al. also developed yet another C-terminal radioimmunoassay using a polyclonal antibody raised against PTHrP 109-138 and were able to detect high levels of PTHrP in the circulation of renal failure patients with MAH [54]. An ultrasensitive multiplex two-site immunoassay has recently been reported capable of simultaneously measuring several circulating forms of PTHrP with a limit of detection (LODs) of 150 aM (~1000 fold lower than current immunoradiometric assay) [55]. The clinical value of such ultrasensitive multiplex two-site immunoassay for the diagnosis and prognosis of cancer patients needs further evaluation in well-established populations.

Current therapies in MAH in individuals suffering from moderate to severe hypercalcemia are aimed at lowering serum calcium levels, and specifically act by inhibiting bone resorption [45]. Bisphosphonates are the standard of care in the treatment of MAH since their approval in the late 1980s, and include mainly pamidronate and zoledronic acid administered intravenously [45]. In 2014, the monoclonal antibody denosumab, directed against RANKL, was approved for the treatment of bisphosphonate refractory hypercalcemia [56]. Even though these currently approved therapies can successfully control blood calcium levels and markedly decrease the symptoms of hypercalcemia, such modalities have had little effect on the patients' mortality associated with the underlying malignancy [45]. However, in the case of HHM, all therapies mentioned above do not target the primary underlying cause of hypercalcemia, namely PTHrP, and it is therefore not yet established whether therapies targeting PTHrP will have an additional benefit. Ogata infused chimeric anti-PTHrP antibodies into nude mice transplanted with PTHrP-secreting human cancer tissues and found this treatment resulted in a

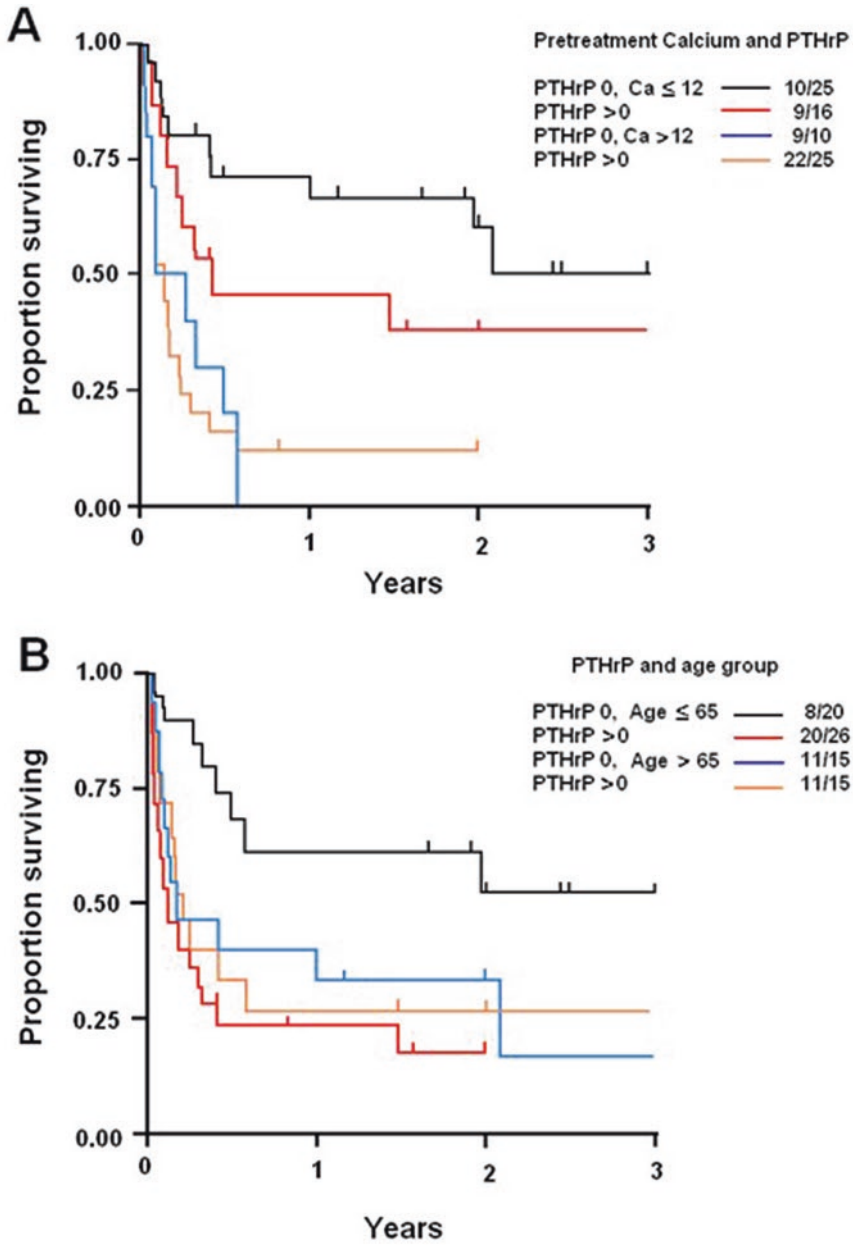

Fig. 4 Serum levels of PTHrP and survival of patients with MAH. (**a**) Survival in 76 hypercalcemic cancer patients, by PTHrP status and pretreatment calcium levels. Numbers shown in the inset are total numbers of deaths/number of patients at baseline. Numbers of patients at risk were 40 at 100 days, 22 at year 1, and 3 at year 3. (**b**) Survival in hypercalcemic cancer patients, by PTHrP status and age group. Numbers of patients at risk were 41 at 100 days, 22 at year 1, and 3 at year 3. CA ≤ 12 = pretreatment serum calcium levels 10.3–12 mg/dL; CA > 12 = pretreatment calcium levels >12 mg/dL; PTHrP 0 = PTHrP not elevated; PTHrP >0 = PTHrP elevated. PTHrP and calcium levels are two independent prognostic factors for patient survival, and the effect of PTHrP is only significant in patients younger than 65. (Reproduce from [50])

prompt and sustained decline in serum calcium [57]. This response was accompanied by improvements in food intake, water intake, body weight gain, and general behavior [57]. Compared to the effects of either bisphosphonates or calcitonin, the author found that some beneficial effects of the antibody were independent of blood calcium levels [57]. These additional benefits of using anti-PTHrP antibodies need further investigation.

PTHrP and Cancer Development

The significance of PTHrP expression by different malignancies is likely not confined to MAH. Overexpression of PTHrP in the absence of hypercalcemia is very common in breast, prostate, lung, and colon cancers [50]. In contrast, normal or non-neoplastic tissues express low levels of PTHrP [50]. This correlation between PTHrP expression and tumor progression suggests that it could be mechanistically linked.

PTHrP expression has been shown to be under the control of numerous growth and angiogenic factors such as transforming growth factor beta (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [50]. Various in vitro studies have provided mounting evidence of the multifunctional role of PTHrP in cancer cell biology including regulation of tumor cell growth, differentiation, and invasion as well as regulation of tumor cell survival factors and interference with apoptotic signaling pathways [50].

PTHrP in Breast Cancer Development

BC is a heterogeneous disease characterized by different pathological and molecular subtypes that have different treatment responses and clinical outcomes. Breast cancer receptor status, most commonly defined by estrogen-receptor (ER), progesterone-receptor (PR), and human epidermal growth factor receptor 2 (HER2) status in the clinical setting, is critical for determining therapeutic intervention and prevention strategies. In addition to the receptor status, five distinct human subtypes have been defined based on gene expression profiling. These include the luminal A (ER and/or PR positive and HER2 negative) and B (ER and/or PR positive and HER2 negative/positive), basal-like (ER, PR, and HER2 negative), claudin-low (ER, PR, and HER2 negative), and HER2/ERBB2-positive (ER and PR negative and HER2 positive) tumors [58]. Most basal-like tumors have a 'triple-negative' immunophenotype (defined by absence of ER, PR, and HER2 expression) and their gene expression signatures are similar to those of normal basal/myoepithelial cells [59].

Studies investigating the role of PTHrP in BC progression have reported conflicting results. Our most recent in silico gene expression analyses using 36 public datasets and 5861 patients identified for the first time significant positive correlations between *PTHLH* expression and components of signaling pathways enriched in the triple-negative breast cancer (TNBC) subtypes including mesenchymal and luminal androgen receptor (LAR) subtypes [60, 61]. Notably, TNBC (ER, PR, and HER2 negative) is characterized by aggressive clinical course, increased rate of metastasis, and lack of targeted therapy, highlighting the need for novel prognostic biomarkers and molecular targets for this disease [62–64]. Therefore, we explored the clinical significance of PTHrP in TNBC using a population-based cohort of treatment-naive patients with newly diagnosed TNBC [65]. Immunohistochemical analysis of PTHrP expression in a tissue microarray constructed for 523 TNBC patients from this cohort revealed that PTHrP is overexpressed in 55.2% of TNBC tumors and its overexpression was significantly associated with decreased overall survival [66]. Consistent with our previously published results in the MMTV-PyMT mouse model, we found that loss of PTHrP expression dramatically prolongs tumor latency and slows tumor growth and metastasis [67]. Additionally, we developed a blocking monoclonal antibody against PTHrP and demonstrated that it could inhibit primary tumor growth and lung metastasis in a human BC xenograft model [67].

These data are in line with several studies showing that PTHrP expression in BC is correlated with poor patient survival [68–72]. In contrast, one study has suggested that PTHrP expression decreases the malignant potential of human breast tumors [73]. In this latter study, Henderson et al. carried out a 10-year prospective analysis of consecutive breast patients at the Breast Unit of St. Vincent's Hospital in Australia, and found that positive PTHrP staining was an independent predictor of improved survival, and reduced metastasis at all sites [73]. Similarly, a study from the same group using an MMTV-*neu* BC mouse model found that disruption of PTHrP resulted in higher tumor occurrence [74].

By contrast, additional pre-clinical and clinical studies appear to support a detrimental role of PTHrP in tumor progression. First, one large genome-wide association study (GWAS) has implicated the *PTHLH* gene as a major BC susceptible locus [75]. Second, Kim et al. found that ablation of the calcium-sensing receptor (CaSR) in the MMTV-PyMT mouse model and in BC cell lines inhibited PTHrP expression and slowed tumor cell growth [76]. Their findings convincingly show that the CaSR-PTHrP pathway contributes to the growth of breast tumors in the MMTV-PyMT mice in vivo. They also documented a positive correlation between CaSR and PTHrP mRNA expression not only in this animal model but also in human samples [76]. This is particularly interesting since the MMTV-PyMT mouse model has been shown to share many characteristics of human basal-like cancer [77]. Finally, using RNAseq analyses, another group has shown that overexpression of PTHrP in MCF7 cells downregulates eight pro-dormancy genes likely through calcium signaling pathways in BC [78].

In summary, based on these observations, PTHrP appears to play a crucial role in BC development. The apparent divergent clinical results between the study published by Henderson et al. and other groups, including ours, could be attributed to features unique to this study, such as the ethnicity/race of the patients or the cut-off for PTHrP positivity in IHC staining analysis.

Pthlh Gene Ablation in Mammary Epithelial Cells and Its Consequences on Tumor Initiation, Growth, and Metastasis

PTHrP is expressed in normal mammary epithelial cells and its expression increased during BC development. To unravel the mechanistic role of PTHrP in BC initiation and progression, we ablated the *Pthlh* gene in mammary epithelial cells and examined its consequence on tumor progression in the MMTV-PyMT mouse model.

The MMTV-PyMT transgenic mouse is an excellent model mimicking many of the biological steps of human BC progression. Lin et al. identified four distinct BC stages, which are comparable to the human diseases from the premalignant stage to advanced disease [79]. Polyomavirus middle T antigen (PyMT) is a membrane-attached protein encoded by the mouse polyomavirus (PyV). PyV expresses three T antigens: a Large T (LT, 100-kDa), a Middle T (MT, 55-Kda), and a Small T (ST, 22-KDa), but only MT is the PyV oncogene that targets key cellular regulators promoting uncontrolled cell proliferation [80]. Previously, the MMTV-PyMT mouse model was regarded as being mainly associated with human luminal B subtype characterized by expression of the luminal K8/18, and overexpression of ErBB2 and low levels of ER [81]. However, a recently published paper showed that MMTV-PyMT mouse has heterogeneous transcriptomes with both luminal B and basal-like phenotypes [77]. During tumor progression, in MMTV-PyMT mouse model, ER and PR receptors status were lost whereas the HER2 receptor was overexpressed [79].

PyMT PTHrP$^{flox/flox}$; Cre^{+} mT/mG Mouse Model

MMTV-Cre transgenic mice have been used extensively to obtain consistently high expression of Cre recombinase in the mammary epithelium [82]. To study the function of PTHrP in BC progression, we used a Cre-Lox recombination system to delete exon 4 of PTHrP [83]. Li et al. constructed *Pthrp*$^{flox/flox}$; *Cre*$^{+}$ (KO) tumor-bearing mouse model specifically inactivating the *Pthlh* gene in the mammary epithelium by crossing homozygous (flox/flox) *Pthlh* mice with MMTV-Cre; MMTV-PyMT mice [67].

The availability of the conditional fluorescent reporter mouse model (mT/mG) has also enabled us to trace Cre expression at a cell-specific level [84]. In this model, the mT/mG reporter transgene is driven by a strong ubiquitous promotor (ACTB) from the well-characterized Gt(ROSA)26Sor genomic locus in which transgene expression of tdTomato (a red fluorescent protein) converts to the expression of enhanced green fluorescent protein (GFP) after Cre recombinase-mediated intramolecular rearrangement of the fluorescent protein-encoding transgene [84]. The activation of the GFP marks Cre expressing cells and all their descendants, since the ROSA26 promoter is expressed in all embryonic and adult mouse tissues [84]. We constructed *Pthrp*$^{flox/flox}$; *Cre*$^{+}$ and *Pthrp*$^{wt/wt}$; *Cre*$^{+}$ mT/mG mice models which can exclusively activate Cre expression in mammary epithelium and specifically express membrane-targeted GFP [85]. The advantage of this approach is to distinguish the membrane-targeted GFP from the membrane-targeted red fluorescent backlight of stromal and nonepithelial-derived mammary tissues during tumor progression (Fig. 5) [85]. Moreover, we will be able to trace and enrich the GFP-positive tumor cells from the primary site to the blood and metastatic site. This model will also help us to further investigate the mechanistic link between PTHrP and tumor progression process from initiation to metastasis.

Role of PTHrP in Breast Cancer Metastasis to Bone

Almost 70% of advanced-stage BC patients will develop bone metastasis that is commonly associated with pain, hypercalcemia, and pathologic fractures [43]. In 1889, Dr. Stephen Paget proposed the "seed and soil" hypothesis, which states that disseminated tumor cells ("the seeds") need the proper microenvironment ("the soil") for them to grow [86]. Cancer metastasis is a complex and multi-step process that involves two major factors: tumor cells and the metastatic site. The tumor cells must detach from the primary tumor, lose their epithelial polarity, invade the basement membrane and extracellular matrix, reach the capillary blood, survive and finally extravasate into a distant site. The metastatic site plays a role as a fertile soil that provides sufficient support for tumor cells to grow. Once metastatic BC cells are in the bone marrow, they can effectively 'hijack' the normal bone homeostatic signals and therefore result in excessive osteoclast activation leading to enhanced bone resorption [43]. Current findings have revealed that there is a 'vicious cycle' in which metastatic cells residing in the bone marrow secrete factors that induce osteolytic bone resorption, and growth factors released from resorbed bone further stimulate tumor growth (Fig. 6).

More than 50% primary BCs and 90% of bone metastasis show strong positivity for PTHrP expression [87]. These have provided the rationale that PTHrP expression in the bone marrow by BC cells promotes bone resorption and tumor cell growth. Due to the complexity of the vicious cycle in the bone metastatic process, the fundamental molecular mechanisms of the role of PTHrP in BC metastasis to bone remain elusive. Previous studies showed that PTHrP is a crucial regulator of bone metastasis and together with other growth factors such as TGF-β and chemokine C-C motif ligand 2 (CCL2) accelerates tumor growth and bone metastasis progression [88, 89]. A series of studies showed that overexpression of PTHrP converted human MCF7 BC cells from a dormant phenotype into a more aggressive metastatic phenotype [90]. Subsequent RNAseq analyses indicated that such overexpression of PTHrP in MCF7 cells downregulated several pro-dormancy genes [78]. The author further confirmed that differential gene expression responses to PTHrP overexpression do not signal through the activation of the cAMP/PKA/CREB pathway mediated by PTH1R [78]. Interestingly, the authors using RNAseq analyses found that the PTHrP overexpression upregulates calcium signaling pathway, but the specific intracellular pathways that mediate these non-PTH1R-mediated actions remain unknown [78]. It has also been shown that CaSR activation stimulated PTHrP production by breast cancer cells in vitro and in vivo which suggests that CaSR acts upstream of PTHrP [76]. These results raised a possibility that PTHrP exerts its osteolytic effect in bone through the activation CaSR signaling,

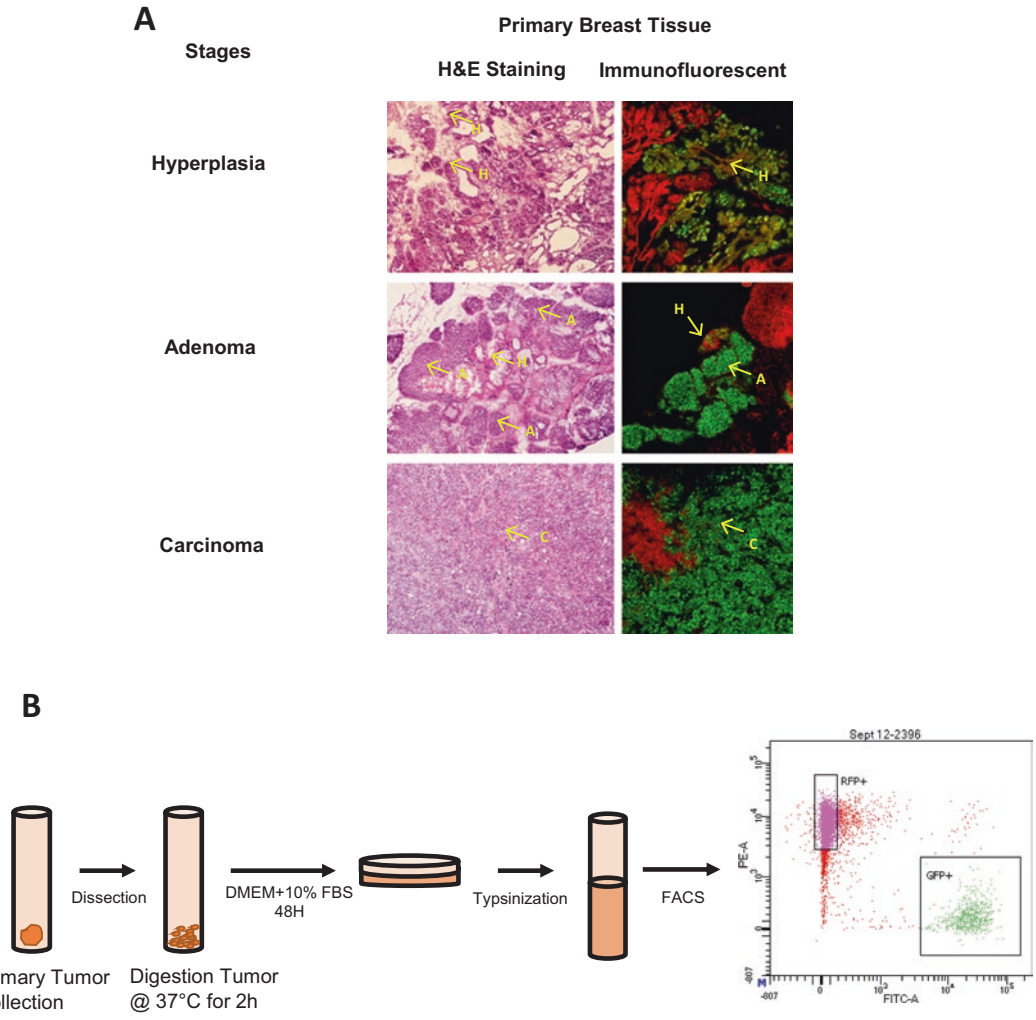

Fig. 5 Cell tracing approaches using PyMT, mT/mG mouse model. (**a**) H&E staining and immunofluorescence staining of tumor tissues at the three stages of tumor development in PyMT flox/flox;Cre + mT/mG mouse model (*H* hyperplasia, *A* adenoma, *C* carcinoma). (**b**) Methods of preparation of tumor cells for fluorescence-activated cell sorting

possibly in a feed-forward loop. Furthermore, how exactly PTHrP produced by tumor cells interacts with other tumor-derived factors into the bone microenvironment is still unclear.

Role of PTHrP in Breast Cancer Metastasis to Brain

BC is the second most frequent cause of brain metastasis after lung cancer, with a risk of 10–16% in advanced BC patients [91]. Previous studies revealed that patients with TNBC or HER2-positive tumors have an increased risk of brain metastasis [91]. Brain metastasis from BC is a catastrophic event associated with a median survival of ~15 months despite treatment [91]. Identification of prognostic biomarkers associated with BC brain metastasis could be beneficial to identify patients at risk and inform appropriate clinical management decisions to improve their survival outcomes. Interestingly, we reported a strong association between high PTHrP expression in archived primary tumors of patients newly diagnosed with TNBC and higher propensity for brain progression [66]. These clinical results

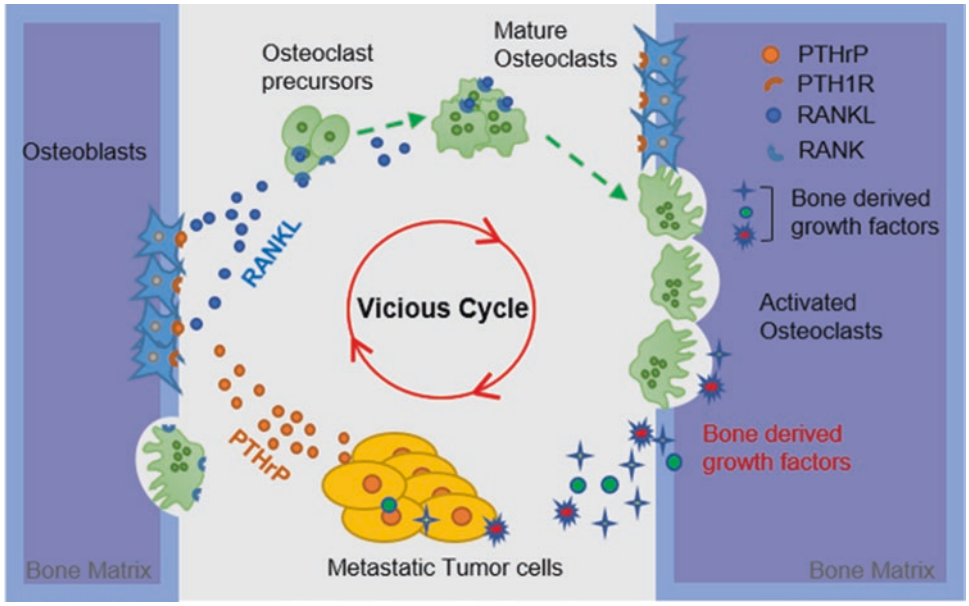

Fig. 6 PTHrP-dependent vicious cycle of bone metastasis. Tumor-derived PTHrP acts in a paracrine manner to stimulate osteoblasts within the bone microenvironment. Stimulated osteoblasts express RANKL, which binds to RANK on osteoclast precursors leading to the formation of multinucleated, bone-resorbing osteoclasts. Finally, osteoclastic bone resorption releases growth factors from the mineralized matrix (bone-derived growth factors), further enhancing tumor growth and survival. This creates a 'vicious cycle' in which tumor-derived PTHrP deregulates bone remodeling and accelerates bone resorption. This self-perpetuating cycle results in increased tumor burden and bone destruction

were also validated using in silico analyses, which showed that *PTHLH* expression positively correlated with signature genes not only involved in bone and lung metastasis in all BC subtypes examined but also, for the first time, correlated with brain metastatic genes: *HBEGF* (heparin-binding EGF-like growth factor) and *ANGPTL4* (angiopoietin-like 4) selectively in TNBC and basal-like subtypes [60]. Collectively, these results reveal for the first time a possible role of PTHrP in TNBC-associated brain metastasis.

Summary and Conclusion

PTHrP was first discovered as the most common mediator of MAH. Subsequently, the discovery of its ubiquitous expression in normal tissues unraveled its role as a physiological autocrine/paracrine regulator. During the bone metastatic process, tumor-produced PTHrP plays a critical role in perpetuating the 'vicious cycle' that was created between the tumor cells and bone microenvironment. Furthermore, mounting evidence appears to indicate that PTHrP regulates most if not all the critical steps of tumor progression within and outside the skeleton. The strong correlation that we identified between PTHrP expression and breast cancer progression in addition to survival in TNBC suggests that PTHrP-targeted therapies representing a promising strategy, which should be further explored in this subtype with limited therapeutic options.

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Targeting DNA Hypomethylation in Malignancy by Epigenetic Therapies

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Abstract

DNA methylation is a chemically reversible epigenetic modification that regulates the chromatin structure and gene expression, and thereby takes part in various cellular processes like embryogenesis, genomic imprinting, X-chromosome inactivation, and genome stability. Alterations in the normal methylation levels of DNA may contribute to the development of pathological conditions like cancer. Even though both hypo- and hypermethylation-mediated abnormalities are prevalent in the cancer genome, the field of cancer epigenetics has been more focused on targeting hypermethylation. As a result, DNA hypomethylation-mediated abnormalities remained relatively less explored, and currently, there are no approved drugs that can be clinically used to target hypomethylation. Understanding the precise role of DNA hypomethylation is not only crucial from a mechanistic point of view but also for the development of pharmacological agents that can reverse the hypomethylated state of the DNA. This chapter focuses on the causes and impact of DNA hypomethylation in the development of cancer and describes the possible ways to pharmacologically target it, especially by using a naturally occurring physiologic agent S-adenosylmethionine (SAM).

Keywords

DNA methylation · Epigenetics · S-adenosylmethionine

Introduction

DNA methylation is a process by which methyl (–CH3) groups are covalently attached to the specific nucleotides on the genome by the action of DNA methyltransferase (DNMT) enzyme [1]. It is an evolutionarily ancient mechanism involved in a wide variety of functions that include the maintenance of genomic integrity and stability, gene regulation, genomic imprinting, cellular differentiation, and protection against foreign DNA [2–7]. In mammalian organisms, methylation of DNA predominantly takes place on the fifth position of cytosine (C) that resides before guanosine (G) nucleotide in sites designated as the CpGs [8]. The distribution and pattern of methylation at the CpG sites are not even across the genome [9]. The CpGs are densely distributed near the gene promoters (in regions called CpG islands) where most of the cytosines are preferentially unmethylated in normal condition [10]. In contrast, methylation is more prevalent on the cytosines

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residing in the repeat sequences and intragenic regions where CpG density is low [11]. In cancer and other pathological conditions, the DNA methylation pattern changes. This change can be in both directions, i.e., both hypermethylation (the condition with increased methylation) and hypomethylation (the condition with decreased methylation)-mediated abnormalities are seen in cancer [1]. Research over the past few decades heavily focused on DNA hypermethylation, and two drugs targeting hypermethylation have been approved by the Food and Drug Administration (FDA) for the treatment of several types of cancer [12, 13]. In contrast, targeting DNA hypomethylation remained less explored. This chapter describes the current state of knowledge on DNA hypomethylation-mediated abnormalities in cancer, especially focusing on the approaches that can be used to target them to reduce cancer-associated morbidity and mortality. The relationship between DNA hypomethylation and cancer stem cells is also discussed.

DNA Hypomethylation and Cancer

DNA hypomethylation refers to the loss of CpG methylation at a specific site on the DNA which is otherwise methylated in a normal state [11]. In the context of the whole genome, the term DNA hypomethylation is used to describe a decrease in the percentage of methylated cytosines compared to the unmethylated ones [11]. There are two types of DNA hypomethylation abnormalities seen in cancer: (1) global hypomethylation; (2) site-specific focal hypomethylation.

Loss of methylation of DNA was the first described epigenetic abnormality in cancer which was reported by two different laboratories in 1983. Feinberg and Vogelstein showed hypomethylation of specific regions on the genome of cancer cells [14, 15]. They used DNA from normal and cancer cells that were digested by methylation-sensitive restriction enzymes for Southern blotting and found a significant decrease in methylation in case of the cancer cells compared to their normal counterpart [14]. Around the same time, by high-performance liquid chromatography (HPLC), the Ehrlich lab showed that the overall level of DNA methylation was considerably decreased in various types of cancer compared to normal tissues [16]. They also showed that the level of CpG methylation was significantly lower in the metastatic neoplasms compared to primary tumors, suggesting the possible association between loss of methylation and tumor metastasis.

Even though DNA hypomethylation was identified before any other epigenetic alterations, it remained the least explored for decades and was often overshadowed by DNA hypermethylation. One of the reasons behind the preference of targeting hypermethylation over hypomethylation of DNA has been a previous bias during the design of an experiment which focused on the sites that are normally unmethylated but becomes methylated in cancer [17]. In that case, there is no chance to observe any decrease in methylation because the sites are already unmethylated. However, with the advancements in high-throughput technologies, it has become apparent that hypomethylation is also a major player in cancer [18–20].

Factors Contributing to DNA Hypomethylation

DNA methylation is a tightly regulated process, and loss of methylation may be contributed by different factors. Some of the most prominent ones are described below.

Methyl Group Metabolism and Dietary Insufficiency

The major component that may act as a limiting factor for methylation of DNA is the universal methyl group donor S-adenosylmethionine (SAM) [21]. The availability of SAM is dependent on dietary methionine as well as recycled methionine obtained from one-carbon metabolism [21]. It has been known for decades that dietary

deficiencies of the methyl group donor may have implication in the development of cancer [22]. Prolonged dietary methyl deficiency in rats has shown to induce global hypomethylation which ultimately contributed to the initiation of hepatocarcinogenesis [23]. The recycling of methionine to produce SAM also requires several vitamins like B_6, B_{12}, and folic acid, and it is important to maintain the level of these vitamins to avoid any abnormal alteration in DNA methylation [24]. Imbalance in any one of these Vitamins may cause SAM deficiency. Several polymorphisms found within the genes (methylenetetrahydrofolate reductase, *MTHFR*; methionine synthase, *MTR*; methionine synthase reductase, *MTRR*; and cystathionine β-synthase, CBS) encoding enzymes involved in the recycling of methionine also showed association with increased risk of cancer [25]. Moreover, the rapidly proliferating cancer cells need more methionine to keep up with the needs of the cells which gets exhausted as cancer progresses [26]. As a result, there is a decrease in the level of SAM which might cause hypomethylation in the cancer genome.

Alteration in DNA Methylation Writers and Erasers

Even though DNA methylation is a stable epigenetic modification, however, the process can be reversible. There are specific enzymes that can mediate the addition and removal of the methyl groups. The DNMT enzymes are involved in the addition and maintenance of methyl group and as such known as the 'writers' of methylation [1]. There are three major DNMTs found in mammalian organisms: DNMT1, DNMT3A, and DNMT3B [27]. In recent years, several enzymes have been identified which can directly or indirectly cause demethylation of DNA, and hence they are called the 'erasers' of methylation [28–32]. Changes in the level and activity of the DNA methylation writers and erasers also contribute to the induction of DNA hypomethylation. In vitro studies have demonstrated that loss of a single or combination of DNMTs can cause hypomethylation as well as chromosomal instability [33]. Similarly, the *Dnmt*-deficient mice showed global hypomethylation which increased the incidence of oncogenesis in vivo [34, 35]. Alteration in the activity of the DNMT enzymes has also been shown to cause hypomethylation of the repetitive sequences in mice [36, 37]. Such loss of methylation of the repetitive elements may have detrimental consequences, which is discussed in the next section of the chapter. In patients with instability-facial anomalies (ICF) syndrome, mutations in *DNMT3B* gene alter the enzymatic activity of its encoded protein which in turn cause hypomethylation at satellite repeats on chromosomes 1, 9, and 16, ultimately leading to chromosomal rearrangements [38, 39]. Increased expression of methyl-CpG-binding domain protein 2 (MBD2), which also possess DNA demethylation/eraser activity [40], showed correlation with DNA hypomethylation [41, 42].

Contribution of Other Epigenetic Factors and Modifiers

Since there is cross talk between DNA methylation and other epigenetic mechanisms like chromatin remodeling and histone modification, the roles of these factors in mediating DNA hypomethylation cannot be overruled. Loss of histone 4 monoacetylation at lysine 16 (H4K16ac) and trimethylation of histone 4 at lysine 20 (H4K20me3) showed association with the hypomethylation at the repetitive sequences of DNA [43]. Histone modifications may also have an indirect effect on DNA hypomethylation through the alteration of chromatin architecture [44]. It has been shown that loss of methylation at H3K9, a modification which is typically associated with heterochromatin establishment and maintenance [45], caused genomic instability [46]. Such disruption of chromatin architecture may have implications in changing the methylation pattern of the DNA.

External Insults

DNA hypomethylation can be mediated by different environmental stressors like exposure to exogenous chemicals and radiation as well as pathogen infection. Koturbash et al. have shown an association between radiation-induced DNA hypomethylation and cancer [47]. They found that majority of the radiation-induced lesions in mice were repaired a month after the insult, but the level of DNA hypomethylation remained the same which was speculated to cause tumorigenesis through the induction of genomic instability. It has been demonstrated that exposure to carcinogens such as benzopyrene and arsenic can cause the induction of DNA hypomethylation [48, 49]. Moreover, viral as well as bacterial infection may also induce hypomethylation of DNA [50, 51].

Impact of DNA Hypomethylation

Some of the important consequences of DNA hypomethylation are summarized below.

Activation of Repetitive Elements

DNA hypomethylation may induce the activation of transposable elements [52] which may induce insertional mutagenesis [44]. Recent studies have demonstrated that promotor hypomethylation activates LINE1 expression in cancer [53], which enables their subsequent retrotransposition. For example, somatic insertion of LINE1 element within the *APC* gene in one allele along with a point mutation in the other allele has been implicated in colorectal tumorigenesis through the two-hit pathway [54]. Once activated, the repetitive elements can also facilitate the expression of other oncogenes and thereby promote tumorigenesis. For example, DNA hypomethylation-induced expression of the LINE-1 transcripts in chronic myeloid leukemia (CML) showed association with the upregulation of the *c-MET* oncogene [55].

Transcriptional Activation of Cancer-Related Genes

Pioneering studies have shown that the promoter of urokinase plasminogen activator (*PLAU*, also called *uPA*) is hypomethylated in cancer which is responsible for its increased gene expression in relatively more aggressive, hormone-insensitive breast cancer cell lines [56]. This was one of the initial studies that provided the proof-of-concept that site-specific focal hypomethylation contributes to cancer progression and metastasis. Other cancer-related genes that showed hypomethylation-mediated activation of gene transcription include heparanase (*HPSE*), cadherin 3 (*CDH3*), breast cancer-specific gene 1 (*BCSG1*), S100 calcium binding protein P (*S100P*), *maspin* (also known as *SERPINB5*), N-acetyltransferase 1 (*NAT1*), pro-opiomelanocortin (*POMC*), related RAS viral oncogene homolog (*R-RAS*), claudin 4 (*CLDN4*), ubiquitin C-terminal hydrolase L1 (*UCHL1*, also known as *PGP9.5*), mesothelin (*MSLN*), *trefoil factor 2* (*TFF2*) [57–64]. Recent epigenome-wide association studies (EWAS) by our group have revealed the presence of hypomethylated sites at the promoters of several oncogenes and prometastatic genes [65, 66]. We have also shown that treatment with the FDA-approved demethylating agent decitabine (5-aza-2′-deoxycytidine) caused hypomethylation-induced expression of *PLAU* and several other prometastatic genes, and transformed less aggressive breast cancer cells (MCF-7, ZR-75-1) into more aggressive ones [67]. This further verified the notion that hypomethylation is involved in metastasis, and careful considerations should be taken while using demethylating agents to treat cancer.

Genomic Instability

Genomic instability is a common characteristic of cancer cells, which includes structural variations in the genome like increased tendencies of base pair mutation, microsatellite instability, and variability in the structure and number of

chromosome (chromosome instability) [68]. In the late 1990s, Chen et al. have shown that murine embryonic stem cells devoid of *Dnmt1* gene predominantly increased the rates of deletion mutations which ultimately led to chromosomal instability [69]. This demonstrated the importance of DNA methylation in the maintenance of genomic stability in mammals. Later on, by generating a mouse model with hypomorphic *Dnmt1* allele that caused global hypomethylation, Gaudet et al. have shown that the mutant mice had increased susceptibility to develop T-cell lymphomas associated with increased chromosomal instability [34].

The first link between DNA hypomethylation and genomic instability in human was reported in human lymphoblastoid cell lines [70]. Further studies have demonstrated that demethylation of classical satellite 2 (Sat2) heterochromatic regions of chromosomes 1 and 16 caused nonclonal rearrangements in lymphoblastoid cells [71]. Since then many studies have demonstrated the association between hypomethylation and genome instability in human cancer. For example, in hepatocellular carcinoma (HCC), hypomethylation of Sat2 sequences showed association with chromosome 1 copy number gain [72]. In urothelial cancer, hypomethylation of Sat2 and Sat3 repeats is associated with loss of heterozygosity (LOH) at chromosome 9 [73]. Cadieux et al. have shown that Sat2 hypomethylation in primary human glioblastomas caused alterations of copy number at the adjacent euchromatin regions [74]. In prostate cancer, hypomethylation is associated with the alterations in chromosome 8 [75].

Cancer Stem Cells and DNA Hypomethylation

Cancer stem cells (CSCs) are defined as a small but unique subpopulation of cells within tumors that have the ability of self-renewal and differentiation [76, 77]. Since they share similar characteristics like the stem cells, they are designated as the CSCs. However, what makes CSCs unique is that they are tumorigenic and can form tumors upon transplantation into an animal. The first modern evidence of the existence of CSCs came in the mid-90s from a study related to human acute myeloid leukemia (AML) [78]. They found a rare population of patient-derived cells that express that the $CD34^{+}/CD38^{-}$ cell surface markers were able to induce leukemia when transplanted into severe combined immunodeficient (SCID) mice [78]. In 2003, two separate studies confirmed the presence of CSCs in solid tumors like breast [79] and brain cancer [80]. Since then, the CSCs have been identified in many other types of cancer [81–91]. Accumulating evidence suggests that the CSCs are resistant to conventional cancer therapies like radiation and chemotherapy [92–95]. They are also involved in metastasis [96]. This makes the CSCs a crucial target for anti-cancer drug development.

A network of signaling pathways including the Sonic Hedgehog (Shh), Notch, and WNT/β--catenin is involved in the regulation of CSC properties [77]. So the inhibition of these pathways may serve as a suitable strategy to target the CSC properties [97]. Interestingly, many of the genes involved in these pathways show epigenetic aberrations in cancer [98, 99]. It has been demonstrated that the promoter of Sonic hedgehog (*SHH*) gene, which encodes the ligand of the Hedgehog signaling pathway, is abnormally hypomethylated in several types of cancer [98–100]. This abnormal DNA hypomethylation leads to the increased expression of this oncogene which is associated with poor overall survival of cancer patients [101]. Furthermore, treatment of human breast cancer cells with FDA-approved DNA methyltransferase inhibitor 5-azacytidine increased the expression of *SHH* gene through promoter demethylation [98]. It has been shown that colon cancer cells (HCT116) grown either in folate-depleted medium or treated with the methotrexate (MTX), which is an antifolate agent, significantly increased the expression of *SHH* gene through promoter hypomethylation [102]. The region on the DNA where this *SHH* hypomethylation takes place also contains a binding site of NF-κB [98]. When the percentage of methylation decreases (hypomethylation state) at the *SHH* promoter, the affinity for NF-κB

binding at this region increases which thereby upregulates the production of *SHH* [98, 99]. Higher expression of the Shh ligand increased the invasiveness of the cells [102]. Hypomethylation was also detected at the promoter of *BOC* gene which encodes the Boc coreceptor involved in the Shh signaling pathway [103]. The expression of Boc is elevated in medulloblastoma which promotes proliferation and subsequent progression from an early to a more advanced stage of tumor through the increase of DNA damage [104]. Similarly, the promoter of t-cell factor *TCF3*, a component of the WNT/β-catenin signaling pathway, has been shown to be hypomethylated in colorectal cancer [105].

Taken together, these studies suggest that DNA hypomethylation plays a key role in maintaining CSC properties. In this regard, we propose that treatment with a methylating agent would likely reverse the stemness of the cancer cells where abnormal hypomethylation is present. It should also be noted that many of the methylation defects are not universal for all types of cancer. The same gene that is abnormally hypermethylated in one cancer can be hypomethylated in another which warrants additional studies to fully elucidate the underlying molecular mechanisms for these selective changes in different malignancies.

DNA Hypomethylation in Diagnosis and Prognosis of Cancer

There is a growing interest in the identification of epigenetic signatures as biomarkers for the early diagnosis and prognosis of cancer. One of the advantages of this strategy is that both DNA and its methylation signatures are quite stable and they can also be extracted from the formalin-fixed, paraffin-embedded samples [106]. Therefore, the limitations related to sample quantity and quality for diagnosis can be mitigated.

Since there is focal hypomethylation of oncogenes in cancer, we and others have reasoned that these specific regions can be exploited as clinical biomarkers for diagnosis and prognosis of different stages of the disease. One of the classic examples in this regard is hypomethylation at the promoter of *PLAU*. Our group was the first to report that there is a reciprocal correlation between aggressiveness of breast cancer and the level of hypomethylation at the *PLAU* promoter [56]. We found that the percentage of methylation decreased with the advancement of breast cancer to a higher histological grade. This suggested that *PLAU* promoter hypomethylation can be used as a biomarker for early detection and aggressiveness of breast cancer [56]. A similar pattern of *PLAU* promoter hypomethylation was also observed between benign prostate hyperplasia and prostate cancer where the methylation level decreased with the advancement of the disease [107]. Hypomethylation-mediated activation of *DDX43* (also known as *HAGE*) gene that encodes cancer testis antigens (CTA) protein showed significant association with disease progression and poor patient outcome in CML patients [108]. Furthermore, *DDX43* promoter hypomethylation also correlated with poorer response to imatinib or interferon treatment in CML patients. This indicates that DNA hypomethylation can also be used for patient stratification before deciding a therapeutic intervention.

It has been demonstrated that hypomethylation of Sat2 repeats in the juxtacentromeric region showed strong association with ovarian cancer progression and mortality and therefore can be used as a marker of poor prognosis [109]. In urothelial carcinoma, Sat2 and Sat3 hypomethylation showed significant correlation with tumor grade and invasiveness of cancer [73]. Hypomethylation of LINE-1 showed association with better prognostic outcome in urothelial carcinoma patients [110]. Using the combined bisulfite restriction analysis (COBRA) PCR assay, Tangkijvanich et al. have shown that serum LINE-1 hypomethylation may serve as a prognostic factor for decreased overall survival in HCC [111].

Another attractive method for early diagnosis of cancer that has been recently demonstrated by us and others is the use of DNA methylation signatures found in blood [112, 113]. During the progression of cancer, the DNA methylation

profiles of the host immune cells are altered [114]. Taking advantage of this phenomena, we have identified and validated six hypomethylated CpG sites [cg27182070 (*RPA2*), cg19761014 (*LRRC37B2*), cg16624210 (*TPPP*), cg00481259 (*DECR2*), cg01252526 (*WDR9*), and cg07271186 (TRY2P)] from the peripheral T-cells of breast cancer patients that can be used as an early detection biomarker [112].

Targeting Hypomethylation

It is obvious that DNA hypomethylation is a crucial player involved in upregulating the expression of many prometastatic genes as well as in the induction of genomic instability in cancer. Since DNA methylation-mediated changes are reversible [115], targeting hypomethylation may serve as a suitable anti-cancer therapeutic strategy. Despite the identification of the role of DNA hypomethylation over three decades ago, there is still no approved agent targeting DNA hypomethylation. On the other hand, many drugs have been developed to target DNA hypermethylation, and two of them are already approved by the FDA. This further emphasizes the fact how DNA hypomethylation remained neglected over the years. Our group has been exclusively focused on targeting DNA hypomethylation as an anti-cancer therapeutic strategy by using SAM. In the following section we will discuss on the effect of SAM treatment in cancer.

S-Adenosylmethionine as a Blocker of DNA Hypomethylation in Cancer

SAM (also known as AdoMet) is a naturally occurring sulfonium compound available in all living cells where it plays role in biochemical processes like transmethylation, transsulfuration, and aminopropylation [116]. Italian biochemist Giulio Leonardo Cantoni initially discovered it in the early 1950s [117].

SAM has a unique chemical structure with a high-energy sulfonium moiety attached to three carbon atoms that are susceptible to nucleophilic substitution [118] (Fig. 1). This renders SAM the ability to donate methyl ($–CH_3$), adenosyl, and aminopropyl groups in different cellular processes. Because of its highly reactive nature, SAM can meditate cofactor functions in different biochemical reactions [118]. In animals, a major portion of the SAM is used in transmethylation pathways where the methyl groups are transferred to different acceptor molecules like DNA, RNA, proteins, and lipids [119, 120]. SAM also donates the aminopropyl groups to produce the polyamines required for cell growth via the aminopropylation pathway [118]. By the transsulfuration pathway, SAM is converted to cysteine which then produces anti-oxidants like glutathione and taurine [121]. SAM also donates the adenosyl portion as well as the $–NH_2$ group for the synthesis of biotin [122]. Taken together, it is obvious that SAM plays a crucial role in the maintenance of cellular function, and abnormalities in SAM metabolism may give rise to different pathological conditions [118]. As such, SAM has been used to treat different diseases. It is approved as a nutraceutical agent for depression, fibromyalgia, osteoarthritis, as well as for diseases related to the liver [116]. Our group was one of the pioneers to test whether DNA hypomethylation-mediated abnormalities of cancer can be reversed by using SAM [123]. Since then, the anti-cancer effect of SAM has been tested both in vitro and in vivo using rodent models in different cancer which is summarized in Table 1.

Suppression of Proliferation and Enhancement of Apoptosis by SAM-Treatment

Uncontrolled cell growth along with suppression of apoptotic cell death is a major characteristic of cancer cells, which has led to the development of several anti-cancer drugs targeting tumor cell proliferation. It has been shown that treatment with SAM can inhibit cell proliferation in vitro in different types of malignancies like breast cancer [124–126], prostate cancer [66], liver cancer [129], colorectal cancer [133], gastric cancer [131], and osteosarcoma [65, 136]. Our recent studies have demonstrated that daily

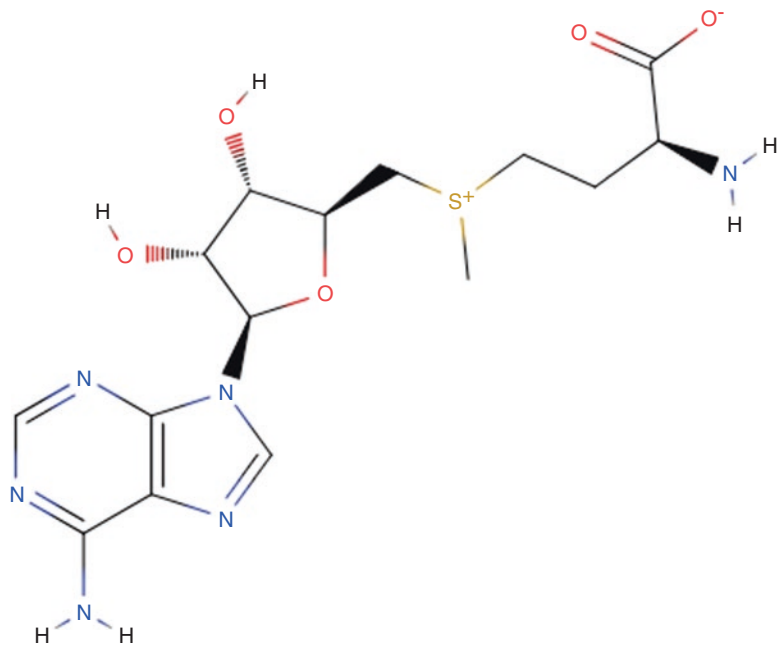

Fig. 1 Chemical structure of S-adenosylmethionine ($C_{15}H_{23}N_6O_5S^+$). The structure was generated using MolView (http://molview.org)

administration of SAM by oral gavage caused a dose-dependent decrease in tumor volume in a xenograft model of breast cancer [124]. Similarly, SAM treatment reduced tumor loads in animal models of gastric and colon cancer [131, 134].

It is also known that rapidly proliferating cancer cells need compact polypeptides known as growth factors for cell proliferation. Therefore, these polypeptides are often targeted by many anti-cancer therapies. In prostate cancer and osteosarcoma cells, we have shown that SAM treatment decreased the expression of genes encoding important growth factors like transforming growth factor β (*TGF-β*), vascular endothelial growth factor (*VEGF*), platelet-derived growth factor alpha (*PDGFA*) [65, 66]. In addition, SAM treatment attenuated key survival pathways mediated by ERK, β-catenin, and signal transducer and activator of transcription 3 (STAT3) in different cancer cells, which also confirms the anti-proliferative effect of SAM [66, 134, 136].

SAM treatment increases both gene and protein expression of dual specificity phosphatase 1 (DUSP1) which is an inhibitor of ERK [137]. In proliferating cancer cells, active ERK1/2 causes phosphorylation at the Ser296 residue of DUSP1 and thereby facilitates its proteasomal degradation via SKP2-CKS1 ubiquitin ligase [138, 139]. It has been suggested that SAM treatment protects DUSP1 from undergoing proteasomal degradation [137].

EWAS using Illumina methylation 450 K array revealed that SAM treatment caused hypermethylation at the promoter of *STAT3* gene which in turn caused a reduction of its gene expression [66]. In gastric and colon cancer cell, SAM treatment reversed the promoter hypomethylation state of proto-oncogenes *MYC* and *HRAS*, which in turn decreased their gene expression [132].

In vitro experiments by us and others have suggested that SAM treatment significantly increases the percentage of apoptotic cell death in different types of cancer and arrests the cancer cells at the G_2M phase of the cell cycle progression [65, 66, 124, 136]. At the molecular level, SAM treatment reduces the expression of anti-apoptotic protein BCl-2 and increases the expression of the proapoptotic Bcl-2-associated X (BAX) protein [124, 136]. This might explain the increase in apoptosis upon SAM treatment. In addition, SAM treatment reduces the expression of cyclin D and E and increases the expression of p53, p21, and p27 [136, 140].

Table 1 Summary of anti-cancer effect of SAM in vitro and in vivo

Cancer type	Effect of SAM treatment	Reference
Breast	Decreased cell proliferation, invasion, migration, colony formation in vitro Increased apoptosis Combination of SAM with other known chemotherapeutic agents (decitabine, doxorubicin) showed a better anti-cancer effect than single-agent treatment in vitro Reduced MDA-MB-231 xenograft tumor volume and metastasis in vivo upon daily supplementation by oral gavage	[124–126]
Prostate	Decreased cell proliferation, invasion, migration, colony formation in vitro Reduced skeletal lesion when SAM-treated PC-3 prostate cancer cells were injected into the tibia of immunocompromised mice	[66, 127, 128]
Liver	Decreased cell proliferation, invasion, colony formation in vitro	[129]
Lung	Combination of SAM with 5-fluorouracil (5-FU) significantly decreased cell proliferation by restoring the levels of DNMTs which are otherwise downregulated by 5-FU monotherapy in vitro	[130]
Gastric	Decreased cell proliferation, colony formation in vitro Reduced SGC-7901 xenograft tumor volume in vivo upon administration of SAM through intraperitoneal injection	[131, 132]
Colorectal	Decreased cell proliferation, invasion, migration in vitro Increased apoptosis Reduction of inflammation-induced colon cancer in vivo Inhibited the metastatic spread of colon cancer cells in the liver in vivo	[133–135]
Osteosarcoma	Decreased cell proliferation, invasion, migration, colony formation in vitro Increased apoptosis Reduced skeletal lesion and lung metastasis when SAM-treated LM-7 osteosarcoma cancer cells were injected in immunocompromised mice via intratibial and intravenous routes, respectively	[65, 136]

The anti-tumor effect of SAM can be partly explained from its unique biochemical structure. SAM can donate methyl groups via the transmethylation pathway which can reduce the hypomethylation-mediated genomic instability seen in cancer. It can produce anti-oxidants via the transsulfuration pathway which may prevent the development of cancer. However, SAM can also donate the aminopropyl groups for polyamine biosynthesis (Fig. 1). Polyamines are involved in the promotion of proliferation in both preneoplastic and neoplastic cells [141]. In rapidly proliferating cancer cells, the level of endogenous SAM goes down favoring polyamine synthesis [142]. Feo et al. showed that exogenous administration of SAM inhibited the activity of a key enzyme of polyamine biosynthesis pathway known as ornithine decarboxylase (ODC), which in turn reduced in the development of nodules in a rat model of liver cancer [142]. Therefore, an overall reduction in tumor volume is observed upon exogenous administration of SAM.

Attenuation of Cancer Cell Invasion and Metastasis upon SAM-Treatment

Tumor-associated metastasis is the most common cause of cancer-related mortality in humans [143]. It is a multi-step process driven by different types of growth factors and proteases that can enable the tumor cells to break down the extracellular matrix (ECM) and migrate into different tissues via the lymphatic system and circulation [143, 144]. The plasminogen activator (PA) system plays a central role in this process [106]. In cancer cells, hypomethylation-mediated upregulation of *PLAU* gene results in the increased production of

uPA protein which binds to its receptor (uPAR) and thereby activates plasminogen to plasmin. Once activated, plasmin can initiate a cascade of proteolytic events to cause the degradation of ECM components [106]. Our group was the first to show that epigenetic targeting of *PLAU* promotor hypomethylation through the use of methylating agent like SAM can reverse its transcriptional state [123]. We found that SAM treatment could downregulate *PLAU* and matrix metalloproteinase 2 (*MMP2*) expression and thereby reduced the invasiveness of the cancer cells as determined by the transwell Boyden chamber invasion assay [123, 127]. These effects were also confirmed by several other research groups working on different types of cancer [131, 133]. We have also shown that oral administration of SAM reduces the metastatic burden of orthotopically implanted MDA-MB-231 breast tumor cells in different peripheral tissues like lung, liver, and spleen of immunocompromised mice [124]. Microarray-based gene expression analysis of the MDA-MB-231 transcriptome revealed that treatment with SAM significantly reduced the expression of several prometastatic and epithelial-mesenchymal transition (EMT) pathway genes [124]. In another study, Tomasi et al. have shown that oral administration of SAM reduces the ability of the colon cancer cells to metastasize to the lung [135]. They have further demonstrated that SAM treatment increases the expression of microRNA-34a and b (miR-34a and miR-34b), which in turn downregulates the IL-6 signaling pathway and thereby decreases the metastatic potential of the cancer cells.

Transcriptome analysis of liver and prostate cancer cells upon SAM treatment also showed downregulation of genes related to cell migration, metastasis, and angiogenesis [128, 129]. Consistent with these observations, methylome analysis upon SAM treatment showed hypermethylation at the promoters of several oncogenes and prometastatic genes [65, 66, 129]. In prostate cancer and osteosarcoma, we showed that inoculation of SAM-treated cells into the tibia of immunocompromised mice reduced skeletal lesion formation at the bone which is a major site of metastasis in many cancers [65, 66]. Sahin et al. have shown that SAM treatment inhibited endothelial cell proliferation which is suggestive of the anti-angiogenic effect of SAM [145].

Combination of SAM with Other Anti-cancer Agents

Since SAM shows little to no toxicity, several groups have tested the anti-cancer properties of SAM in combination settings with currently approved chemotherapeutic agents. Ilisso et al. investigated the effect of SAM in combination with Doxorubicin on different breast cancer cell lines [126]. Doxorubicin is a classic chemotherapeutic agent used for patients with breast cancer. Even though it is highly effective as an anti-cancer agent, it also elicits several side effects including cardiomyopathy, alopecia, vomiting as well as resistance to therapy [146]. The authors have reasoned that combination of SAM and doxorubicin will allow lowering the concentration of doxorubicin and thereby reduces doxorubicin-associated side effects [126]. They found a significantly synergistic anti-proliferative effect following combination therapy with doxorubicin and SAM in hormone-dependent breast cancer cells. This highly significant effect on cancer cells following combination therapy was shown to be due to the increase in the percentage of apoptotic cells.

Using human A549 lung cancer cells, Ham et al. showed that the anti-cancer effect of another agent, 5-fluorouracil (5-FU), is markedly enhanced when used in combination with SAM [130]. They have shown that single-agent treatment with 5-FU reduced the expression of DNMTs which is restored upon combination treatment with SAM.

It has been recently demonstrated that SAM in combination with autophagy inhibitor chloroquine (CLC) shows better anti-proliferative effect through the induction of apoptotic cell death [140]. The authors have further shown that the combination treatment synergistically inhibited the phosphorylation of AKT/mTOR kinases which are major survival pathways of the cancer cells.

SAM has also been used in combination with other epigenetic drugs. Previous studies by our group have demonstrated that the FDA-approved DNA methylation inhibitor decitabine undesirably activates the expression of several prometastatic genes [*PLAU*, heparanase (*HPSE*), synuclein-γ (*SNCG*), and C-X-C motif chemokine receptor 4 (*CXCR4*)] along with the activation of tumor suppressor gene like Ras association domain family member 1 (*RASSF1*) and proapoptotic gene *BAX* [67]. We found that inoculation of decitabine-treated MCF7 breast cancer cells into the fat pad of immunocompromised mice showed significantly reduced tumor burden compared to the control group of mice implanted with untreated cells which is consistent with the known tumor suppressive effect of decitabine. However, when the tumors were analyzed after the sacrifice of the animals on week 6, a significant induction of several prometastatic factors like PLAU, heparanase, and CXCR4 was observed in the decitabine-treated group. So, we concluded that decitabine treatment increased the invasiveness of less aggressive MCF7 breast cancer cells. This might be a reason why these drugs are not as effective in solid tumors compared to the liquid tumors for which they are approved. SAM, on the other hand, reduces the expression of several prometastatic genes [123]. Since the cancer cells are heterogeneous, the pattern of DNA methylation abnormalities may be different between the cells within the tumor microenvironment. As such, targeting both hypermethylation (by decitabine) and hypomethylation (by SAM) has been tested as a proof-of-concept, and it was found that the combination treatment synergistically reduces proliferative and invasive capacities of several breast cancer cell lines [125]. It was shown that the combination of SAM and decitabine could inhibit the expression of prometastatic genes like *PLAU* and *MMP2* which are otherwise induced by single-agent treatment by decitabine. Moreover, SAM did not hinder the expression of tumor suppressor genes (*CDKN2AIP* and *p21*) which are normally induced upon decitabine treatment. This implies that SAM affects tumor suppressor genes and prometastatic genes differently. However, the study was limited to a few genes only, and whether a similar effect can be recapitulated in vivo using mouse models of breast cancer still remains to be seen. Nevertheless, the study has opened the door for a novel combinatorial approach using two epigenetic agents that can target various elements of the DNA methylation-mediated abnormalities seen in cancer.

Conclusion and Future Prospective

It is still surprising that more than three decades after its initial identification in 1983, there is still no approved anti-cancer agent that can reverse the DNA hypomethylation-mediated abnormalities in cancer. The recent surge of EWAS has further emphasized that DNA hypomethylation is a crucial phenomenon during cancer progression. Our group and others have shown that methylating agent SAM can reverse the DNA hypomethylation-mediated abnormalities in established cell lines and animal models of cancer without showing any toxic/detrimental effect (Fig. 2). One of the main advantages of SAM is that it is a naturally occurring agent already approved and available as a nutraceutical supplement. In addition, there is a plethora of clinical trials that tested its role in depression and liver diseases, and results from those studies indicate that exogenous SAM is tolerable and non-toxic in clinical settings. Two clinical trials using SAM as an anti-cancer agent are at the early stage of patient recruitment. Results from these studies will further shed light on its true potential as an anti-cancer agent.

Being a pleiotropic agent, SAM can also have effect on the methylation of other molecules like RNA, proteins, and lipids. Currently, not much is known about the anti-cancer effects of SAM on the macromolecules other than DNA. This is an area where further exploration is needed. Moreover, the development of agents with better stability, half-life, specificity, and therapeutic efficacy as compared to SAM is also warranted.

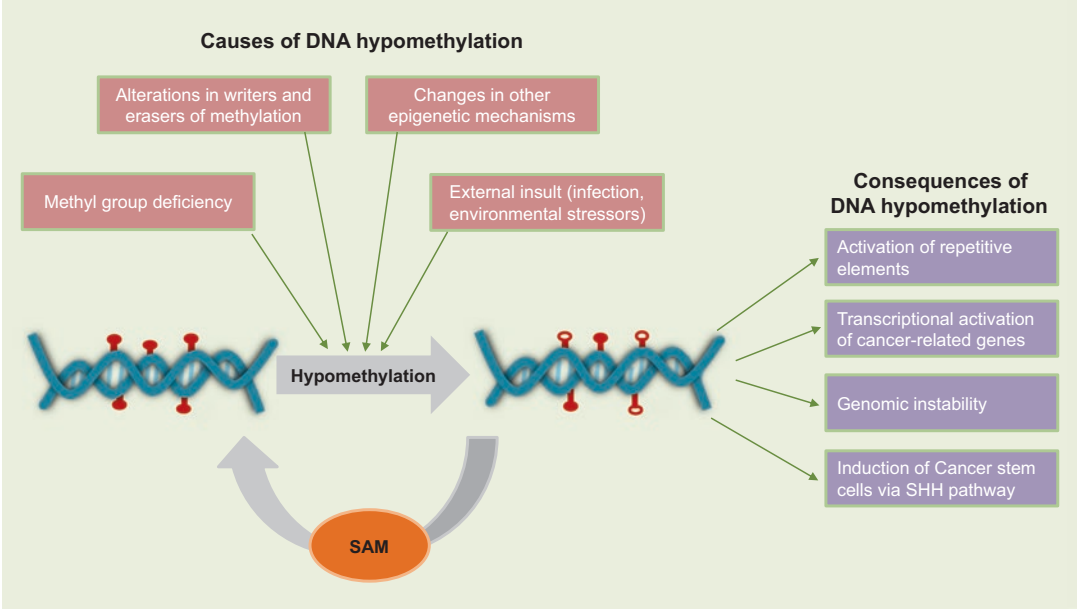

Fig. 2 Causes and consequences of DNA hypomethylation and reversal of the hypomethylated state by methylating agent like SAM

Another exciting avenue that has not been exploited to its full potential is the effect of DNA methylating agent like SAM on the immune system during cancer progression. Li et al. have shown that SAM treatment can reduce inflammation-induced colon cancer in vivo [134]. They have also shown that SAM treatment can reduce the expression of interleukin-6 (IL-6) and IL-10. Furthermore, a recent study has demonstrated that SAM downregulates forkhead box P3 (FOXP3) expression, which is a master regulator of regulatory T-cells (Treg cells) [147]. Accumulating evidence suggests that Tregs suppress the anti-tumor responses and are often associated with poor clinical prognosis [148]. Since SAM functions in modulating the expression of Tregs, it will not be surprising if they are also associated with the immune checkpoint proteins. It would be equally important to see how the combination of therapeutic agents against DNA hypomethylation and immune checkpoint inhibitors acts in blocking tumor growth and metastasis to reduce cancer-associated morbidity and mortality.

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Conflict of Interest The authors have no conflicts of interest to declare.

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Part IV

Targeting the Tumor and Its Microenvironment

Tumor Dormancy and Slow-Cycling Cancer Cells

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Abstract

Cancer cell heterogeneity is a universal feature of human tumors and represents a significant barrier to the efficacy and duration of anticancer therapies, especially targeted therapeutics. Among the heterogeneous cancer cell populations is a subpopulation of relatively quiescent cancer cells, which are in the G0/G1 cell-cycle phase and refractory to anti-mitotic drugs that target proliferative cells. These slow-cycling cells (SCCs) preexist in untreated tumors and frequently become enriched in treatment-failed tumors, raising the possibility that these cells may mediate therapy resistance and tumor relapse. Here we review several general concepts on tumor cell heterogeneity, quiescence, and tumor dormancy. We discuss the potential relationship between SCCs and cancer stem cells (CSCs). We also present our current understanding of how SCCs and cancer dormancy might be regulated. Increasing knowledge of SCCs and tumor dormancy should lead to identification of novel molecular regulators and therapeutic targets of tumor relapse, residual diseases, and metastasis.

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Keywords

Cancer stem cell · Tumor dormancy · Quiescence · Slow-cycling cell · Prostate cancer · Tumor cell heterogeneity · Label-retaining cell · LRIG1 · Cell cycle · scRNA-Seq · Lineage tracing · Prostate stem cell · TGF-beta · Cell-of-origin · Self-renewal · Progenitor · Plasticity · CD44

Introduction: Cellular Heterogeneity in Cancer

Cellular heterogeneity represents an omnipresent feature in human tumors, which contain distinct subsets of cancer cells with diverse morphology, cytogenetic markers, growth kinetics, immunological characteristics, metastatic ability, and sensitivity to therapeutics [1]. Understanding cellular heterogeneity in solid tumors should facilitate development of new diagnostic and therapeutic strategies. The majority of current anti-cancer drugs are anti-mitotic and molecularly targeted agents, which mainly target the differentiated and proliferating cancer cells [2]. However, recent evidence suggests that there is always a population of quiescent, slow-cycling cells (SCCs) that preexist in primary tumors and is less affected by standard treatments. *Experimentally*, reports have shown that SCCs exist in many human cancer cell

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cultures and xenograft tumors [2, 3]. *Clinically*, patients with cancer can develop recurrent and metastatic disease with latency periods that range from years to decades. *Pathologically*, subsets of Ki-67-negative cells, characterizing diminished proliferation, are always observed in virtually all human tumors [4]. These all indicate the existence of SCCs in models as well as patient tumors.

Quiescence and Slow-Cycling Cells (SCCs) in Cancer

Quiescent, slow-cycling cells (SCCs) exist in clinical human tumors [4], and accumulating evidence suggests that SCCs may play a vital role in many aspects of cancer biology. Due to their intrinsic dormancy, SCCs are thought to be insensitive to most current clinical treatments, and thus may contribute to tumor relapse [3]. However, few studies have developed systems to PROSPECTIVELY study SCCs. Upon entering the non-proliferative G0/G1 cell-cycle phase, cells have relatively low metabolic activity in a state of cell-cycle arrest, a term referred to as quiescence. Transforming growth factor-β (TGF-β) is among the many secreted factors that mediate microenvironmental signaling that controls cellular differentiation, proliferation, and survival. TGF-β has been shown to have tumor suppressive properties in that it opposes normal epithelial cell proliferation [5]. TGF-β has been reported to maintain dormancy in stem cell populations in the prostate [6], liver [7], gastrointestinal system [8], and in the bone marrow [9].

In many cancers, a small population of SCCs has been identified in cell cultures, xenografts, and clinical tumors, and SCCs are thought to survive anticancer therapies and contribute to later disease recurrence and metastasis [2–4, 10]. Additionally, SCCs may also have implications in immunotherapy; for example, vaccination with drug-resistant slow-cycling tumor cells caused a reduction in tumor volume and prolonged the overall survival of tumor-bearing mice [11]. Therefore, isolation and functional study of SCCs will be keys to developing future therapies that better target dormant cancer cells to prevent recurrence.

Clinical Evidence for Cancer Dormancy

Although poorly understood, cancer dormancy has been generally classified into two entities: (1) tumor mass dormancy, whereby active cancer cell proliferation is mechanistically opposed by apoptosis, and (2) tumor cell dormancy, characterized by inactive tumor cells that have entered into prolonged G0/G1 cell-cycle arrest [4]. Whereas tumor mass dormancy is limited by factors such as poor neovascularization and susceptibility to immune surveillance, tumor cell dormancy represents a clinically asymptomatic form of dormancy where these cells can become active months to decades later. The major concern of dormant cancer cells is whether they can be revived from an inert state to rapidly growing overt deadly cancers. This raises the question of how to detect dormant cells. The evidence for dormant cancers has been demonstrated by autopsies of patients diagnosed with cancer as well as trauma victims [12]. Other evidence of cancer cell dormancy arises from the findings that disseminated tumor cells (DTCs), which are cells that physically separate from the primary tumor mass and travel to other sites in the body via circulation, have the ability to enter a dormant state and become resistant to therapies [13].

Cancer Stem Cells (CSCs)

The topic of CSCs has been debated by researchers throughout the years mostly because of the lack of the ability to consistently assay and uniformly define these cell populations. Stem cells (SCs) are defined as cells that possess self-renewal and differentiation abilities [14]. Historically, most SCs in adult tissues and organs have been identified by panels of cell surface markers, in vivo lineage tracing, and by their intrinsic quiescent and slow-cycling properties [3]. Additionally, functional assays that measure

drug efflux (e.g., side population) and aldehyde dehydrogenase (ALDH) detoxifying capacity (i.e., Aldefluor) may be used to purify and enrich stem cells [1]. CSC is a functional term and can be most properly defined in functional assays by their ability to re-generate serially transplantable tumors with features of the parent tumor (e.g., cellular heterogeneity and specific cell surface markers) [3]. Several terms, including dormant or quiescent cells, SCCs, and label-retaining cells (LRCs) may be used interchangeably; however, CSCs may not necessarily be included among these cell types. Generally, dormant or residual slow-cycling tumor cells are thought to be a major source of tumor relapse and metastasis, and are therefore an obstacle to therapy. SCCs and CSCs are two "semantic" terms describing two overlapping cancer cell subpopulations in a continuum [2–4]. In other words, some (but NOT all) SCCs may possess both phenotypic and functional properties of CSCs and vice versa, and some (but NOT all) CSCs may be dormant and slow-cycling. For instance, SCCs in some tumors have been shown to possess CSC-related properties, e.g., enhanced tumor-propagating ability, therapy resistance, and promotion of tumor relapse and metastasis [3]. On the other hand, although some CSCs, e.g., the prostate-specific antigen-negative/low ($PSA^{-/lo}$) CSCs [1] and the cluster of differentiation 44-positive ($CD44^{+}$) [15, 16] prostate cancer (PCa) cell populations, are relatively quiescent at the population level, other CSC populations, e.g., the $ALDH^{+}$ cells [1, 3], may be proliferative. This is analogous to the existence of well-established quiescent AND cycling normal SC populations [3]. Much more effort has been devoted to the studies of CSCs than SCCs, as evidenced by identification of CSCs in virtually every tumor system [3, 16–20]. However, SCCs clearly exist in tumors and may play a critical role in regulating tumor cell subpopulation dynamics. For example, a population of slow-cycling melanoma cells bearing a lysine demethylase 5B-high (KDM5B-high) phenotype is required for continuous tumor growth [21], and therapeutically targeting this population can overcome the intrinsic multidrug resistance in melanoma [22]. Our lab is currently cross-examining the inter-relationship between purified PCa SCCs and prostate cancer stem cells (PCSCs) identified using the $PSA^{-/lo}$ phenotype and other markers such as $CD44^{+}$, ALDH-high, and $ABCG2^{+}$ to compare their relative "stemness," aggressiveness, therapy sensitivities, and ability to repopulate recurrent tumors.

Modeling Cancer Cell Dormancy

A recent review by Kester and van Oudenaarden discussed new advances in sequencing technologies used either alone or in combination to predict cellular differentiation trajectories based on single-cell transcriptomics [23]. For example, the techniques described combines single-cell genetic lineage tracing with differentiation trajectory algorithms to reliably capture cell-type heterogeneity to therapy responses. These same technologies can be used to investigate lineage relationship between stem cells and their mature progeny, traced over time. Tracking lineage-related changes in genomic signatures by either introducing specific alterations experimentally (prospective lineage tracing) or following intrinsic non-perturbation processes (retrospective lineage tracing), researchers can differentially determine gene signatures associated with a single founder cell [23]. The utility of dyes into single founder cells along with flow-assisted cell sorting (FACS) has opened many avenues for investigators to more readily study cancer cell initiation, cell-of-origin, and tumor dormancy.

Studies have also been utilizing high-throughput single-cell RNA-Seq (scRNA-Seq) to investigate cellular heterogeneity as progression trajectory in relation to metabolomic changes that occur during chemical reprogramming [24]. Such novel powerful techniques demonstrate that fully differentiated, mature cell progression tracing can be associated with transcriptomic and epigenomic changes that allow cell fate experimental manipulations to be performed prospectively.

Yet another powerful method of studying tumor dormancy is to employ the LRC model. Generally, normal mammalian adult SCs are

slow-cycling, and long-lived cycling SCs have been reported in rapidly renewing tissues such as hair follicles, small intestine, and blood [25]. In practice, label-retaining techniques are frequently employed to study SCs, although this technique is meant to label SCCs rather than SCs. In this technique, tissues (or cells) are first pulsed with a DNA base analog, e.g., 5-bromo-2′-deoxyuridine (BrdU), which is followed by an extended period of chase. Such identified LRC population is often enriched in functional SCs. In addition to DNA analogs, which unfortunately CANNOT be used to purify out LIVE SCCs, other label-retaining techniques (e.g., Tet-controlled H2B-GFP fusion protein and cell membrane labeling dyes such as PKH26) have been developed to identify and purify SCCs for functional studies [26].

Visualizing cell cycle transitions, generally, has proved difficult. A relatively new technique known as the FUCCI (fluorescence ubiquitination cell-cycle indicator) has been developed, which exploits the inversely oscillating levels of two separate cell-cycle licensing factors, fused with either green or red fluorescent probes [27]. In this system, time-lapse video fluorescence microscopy captures the switch from green- to red-emitting signals as the cell cycles from G1 to S/G2/M, respectively [27]. Modified FUCCI-based systems allow for G0/G1-phase cell separation to study dormancy in FUCCI-expressing cell lines, stem cell lineages, and in mouse models [28], allowing the enrichment and isolation of SCCs for studying their involvement in therapy resistance, disease recurrence, and drug screening.

Recently developed 3D cell and tissue culture technologies such as organoid systems have become increasingly efficient in drug development and personalized medicine for primary and metastatic colorectal, pancreatic, prostate, breast, and brain tumors [29]. Organoids are currently used to model mutational processes underlying tumorigenesis, response to immunotherapies, and the contribution of CSCs to tumor growth.

Pharmacokinetic and pharmacodynamic (PK/PD) methods have recently incorporated imaging techniques to assess the delivery and efficacy of fluorescently labeled drugs by in vivo microscopy, e.g., to investigate tumor-stromal signaling, tumor vasculature, and drug efflux capacity of CSCs [30]. Dynamically tracking circulating tumor cells (CTCs) found in the bloodstream has led to the introduction of "real-time" liquid biopsies, a way to routinely monitor cancer progression, relapse, and patient response to therapies with minimal invasion and low risk for effects [31].

Prostate Cancer as a Model of Cancer Dormancy-Related Drug Resistance

Prostate cancer (PCa) is a heterogeneous malignancy. Androgen deprivation therapy (ADT) is the current main therapeutic regimen for advanced PCa patients. However, most treated patients invariably develop the castration-resistant prostate cancer (CRPC). The cell(s)-of-origin for and mechanisms underlying CRPC development and maintenance remain poorly understood. Recently, we have reported a $PSA^{-/lo}$ PCa cell population that exists in primary tumors at low frequency but dramatically increases in high-grade primary tumors and, in particular, recurrent PCa [15]. Importantly, the $PSA^{-/lo}$ population, which expresses stem cell gene signatures and possesses many cardinal SC properties, can function as both cells-of-origin AND tumor-propagating cells in CRPC [1, 15, 32]. The $PSA^{-/lo}$ PCa cell population, compared to PSA^{+} cells, is quiescent, enriched for CSCs that express low androgen receptor (AR), and drug-resistant and tumorigenic [1, 15, 33]. In multiple other cancer systems, studies have also suggested that SCCs can survive anticancer therapies and contribute to later disease progression and metastasis [2–4, 21, 34]. However, no prospective studies have been performed to elucidate the clinical importance of SCCs in PCa, especially in response to ADT and subsequent CRPC development. Therefore, utilizing PCa as a model disease system, we aim to describe the implications of PCa SCCs in more broad terms of cellular quiescence and tumor cell dormancy and apply these concepts

to other cancer types as it relates to therapy resistance and repopulation of primary cancers, disease recurrence, and progression to invasion and metastasis.

Unfortunately, to our knowledge, no systematic and prospective studies have focused on SCCs as potential effectors and mediators of therapy (including castration) resistance in PCa and emergence of CRPC. Moreover, no drugs have been developed as yet to specifically target quiescent PCa cells. As in other tumor systems, there is an urgent need to advance our knowledge of quiescent cell biology and thus provide a strong foundation to develop potential therapeutic strategies to target this obstinate population. One example would be to develop novel treatment options by combining ADT and SCC-specific therapies to eventually prevent/eliminate CRPC in patients, which should ultimately impact PCa patient survival.

LRCs and Normal Mouse and Human Prostate SCs

Adult prostate renews slowly and can undergo multiple rounds of castration-induced regression and testosterone-induced regrowth, attesting the presence and the functional importance of SCs. Since the first report of putative prostate SCs via LRC experiments by Dr. E. Wilson's group, the proximal region (i.e., close to the urethra) of the prostatic tubules [35] and the basal location [36–40] have been proposed to be the niche to maintain the quiescence (mediated by TGF-β) [6] of prostate SCs. By using lineage-tracing techniques, studies have reported the existence of lineage-restricted stem/progenitor cells within both basal and luminal layers of the mouse prostate [41–43], which is further validated by a recently developed 3D organoid system in the human prostate [44]. Importantly, these reports indicate that primitive prostate SCs are generally quiescent in vivo [41, 45]. Our lab, over the years, has studied, and made extensive use of, normal primary human prostate epithelial cells from normal/benign human prostates [1, 16, 46–48]. We recently described a genome-wide transcriptome analysis of human prostatic basal and luminal populations using deep RNA-Seq, and found that basal cells are generally quiescent in situ and molecularly resemble aggressive PCa [47]. Also, we recently developed a feasible 2D culture system to enrich high numbers of human prostate luminal progenitor cells and further showed that these cells could function as a cell-of-origin for PCa [46]. In addition, we have established several complimentary experimental strategies that enable us to purify out LIVE SCCs from human PCa cell cultures and xenograft tumors for probing their functional properties. Importantly, we have also generated unique transgenic mouse models of label-retaining cells (LRCs) to study SCCs in a naïve tumor microenvironment under unperturbed and androgen-ablated conditions. The utility of these techniques will help in determining the inter-relationship between SCCs and CSCs as well as the expression status of the AR in the SCC population and its impact on SCC biology. By performing gene-expression analysis coupled with functional assays, it may be feasible to identify potential therapeutic targets that could lead to the elimination of this "hard-to-kill" population.

PCSCs (Prostate Cancer Stem Cells): Hierarchical Organization and Relative Dormancy

Over the past 15 years, our lab has been meticulously dissecting the FUNCTIONAL heterogeneity in human PCa cells. Our systematic work, which has provided a framework of understanding of PCa cell heterogeneity, has demonstrated that the human PCSC pool largely resides in the undifferentiated $PSA^{-/lo}$ PCa cell population [1, 9, 15, 16, 32, 49]. The $PSA^{-/lo}$ PCSC pool contains multiple subsets of tumorigenic cells [50–53] and, importantly, many $PSA^{-/lo}$ PCa cells and subsets of PCSCs lack appreciable expression of AR and are dormant, which, together, render these cells intrinsically refractory to both anti-androgens and anti-mitotic drugs such as docetaxel and etoposide. Whether SCCs are het-

erogeneous in AR expression and what is the impact of AR status on SCC functions have yet to be investigated. Another area of interest includes the determination of whether PCa SCCs can survive ADT and repopulation CRPC in vitro and in vivo, and characterizing transcriptome changes of LRCs during CRPC emergence.

Many molecular entities and circuits initiate and enforce cancer cell dormancy. For example, LRIG1 (leucine-rich repeats and immunoglobulin-like domains protein 1), known as a pan-ERBB negative regulator, is well established to promote adult stem cell quiescence, especially in epidermis and gastrointestinal system [15, 54–59]. LRIG1 functions as a tumor suppressor in many cancers [58]. Gene-expression profiling reveals LRIG1 enrichment in $PSA^{-/lo}$ PCa cell population [15]. The $PSA^{-/lo}$ cell population harbors highly dormant PCSCs possessing tumorigenic, metastatic, and CRPC-initiating and -propagating properties.

Of great clinical interest is whether a tumor can be contained indefinitely in a dormant, non-malignant state or whether driving SCCs out of dormancy is a better therapeutic approach. There exist several research opportunities to study the differences between persistence of tumor dormancy versus reactivation. Studies have demonstrated that CoCo, a bone morphogenic protein 4 (BMP-4) inhibitor protein secreted by the stroma [10], reactivates dormant breast cancer cells localized to the lungs, providing evidence of DTC escape from dormancy. It was also found that breast cancer cells with low CoCo expression remained dormant [60]. Thus, it is important to develop a systematic functional characterization of SCCs in human PCa cells and xenograft tumors, LRCs in genetic mouse models of prostate tumors, and gene-expression profiling of SCCs in human and mouse PCa to address the clinical implications associated with tumor dormancy.

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Resolution of Cellular Heterogeneity in Human Prostate Cancers: Implications for Diagnosis and Treatment

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Abstract

Prostate cancers have a justified reputation as one of the most heterogeneous human tumours. Indeed, there are some who consider that advanced and castration-resistant prostate cancers are incurable, as a direct result of this heterogeneity. However, tumour heterogeneity can be defined in different ways. To a clinician, prostate cancer is a number of different diseases, the treatments for which remain equally heterogeneous and uncertain. To the pathologist, the histopathological appearances of the tumours are notoriously heterogeneous. Indeed, the genius of Donald Gleason in the 1960s was to devise a classification system designed to take into account the heterogeneity of the tumours both individually and in the whole prostate context. To the cell biologist, a prostate tumour consists of multiple epithelial cell types, inter-mingled with various fibroblasts, neuroendocrine cells, endothelial cells, macrophages and lymphocytes, all of which interact to influence treatment responses in a patient-specific manner. Finally, genetic analyses of prostate cancers have been compromised by the variable gene rearrangements and paucity of activating mutations observed, even in large numbers of patient tumours with consistent clinical diagnoses and/or outcomes. Research into familial susceptibility has even generated the least tractable outcome of such studies: the genetic loci are of low penetrance and are of course heterogeneous. By fractionating the tumour (and patient-matched non-malignant tissues) heterogeneity can be resolved, revealing homogeneous markers of patient outcomes.

Keywords

Prostate cancer · Heterogeneity · Epigenetics · Gene expression

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Prostate Tumour 'Cells' as a Therapeutic Target

Based on the Gleason histological system devised in the 1960s [1], imperfect conclusions about prostate cancer patient outcome can be made. The treatment of the tumour, despite its frequency, has yet to be optimised. Studies designed to decide the best early stage treatment such as ProTect [2] have provided an indication,

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but with a long time scale required to produce a decision. It is however quite clear from another long-term study by ERSPC [3] that early intervention based on screening for prostate-specific antigen (PSA) can have an impact on death rates, with the enduring risk of over-treatment in the absence of good molecular markers for aggressive disease [4]. However, when primary prostate cancer has escaped from the blockade of male sex hormone responses, the full impact of heterogeneity can be seen. The mean survival times, despite new generation drugs like abiraterone, apalutamide and enzalutamide, designed to manipulate androgen responsiveness [5], and optimised taxane treatments which targets dividing cells [6], remain stubbornly at about 2 years [7]. Indeed, many established treatments from other tumour types have only limited efficacy against advanced castration-resistant prostate cancer (CRPC) [8].

There are few simplistic explanations for this apparent resistance. Prostate cancer has historically been considered to be a slow-growing tumour, yet the cancer cells cycle at about the same rates as those from other tumours. A more likely explanation comes from the observation that prostate cancers seem to have a relatively high intrinsic apoptotic rate: resulting in a *net* slower increase in tumour bulk and cell numbers [9]. If this is indeed the case, then there is little justification based on slower cell divisions for the observed resistance to drugs like etoposide and docetaxel, which interfere with the processes of genome and cell duplication, respectively.

We are now firmly in the era of novel, targeted therapies for many cancers (including the prostate), where genomic information is rapidly translated into specific reagents such as antibodies and interfering RNA, which can eliminate the activity of particular enzymatic targets. The issue of heterogeneity directly impinges on the use of these targeted reagents. As discussed below, given the degree of polymorphism in man, and the different types of heterogeneity seen in cancers, can one highly targeted drug ever be designed to treat all patients with even a specific grade of a specific cancer?

Cellular Heterogeneities Within a Prostate Cancer

To a generation of cell biologists brought up on established cell lines, which could be exploited in controlled growth conditions to model disease, the sheer heterogeneity of cell phenotypes present within a single cancer can appear rather daunting. Such mixtures of cells are only too familiar to prostate histopathologists, who have successively refined the original Gleason grading scheme [10]. Increasingly multicellular laboratory studies are now revealing the true roles played by the many cell types which affect the behaviour of the primary tumour cells (secretory epithelial cells for an adenocarcinoma-like prostate cancer). The representations in Fig. 1 illustrate the many cell types to be found within the prostatic normal and tumour microenvironments [11].

The fate of the epithelial cells is intimately linked to the presence of the ancillary cells, a situation which is recapitulated when the primary tumour successfully migrates to a primary and secondary metastatic site. Such tumour spread is frequently associated with epithelial to mesenchymal transition, a transitory state in which a frank epithelial morphology, and the gene expression profile of the cancer cells, changes to one resembling a mesenchymal (stem) cell [12]. Under these conditions a further degree of (morphological) heterogeneity is observed within what is essentially the same tumour cell at the genomic level.

One interesting feature of the tumour microenvironment is the extent to which the tumour epithelium defines the phenotype of the ancillary cells, i.e. the development of a tumour is marked by co-evolution of the primary transformed cell (which we presume to be the epithelial cells in prostate), perhaps with genetic and epigenetic changes in the associated mesenchyme [13, 14].

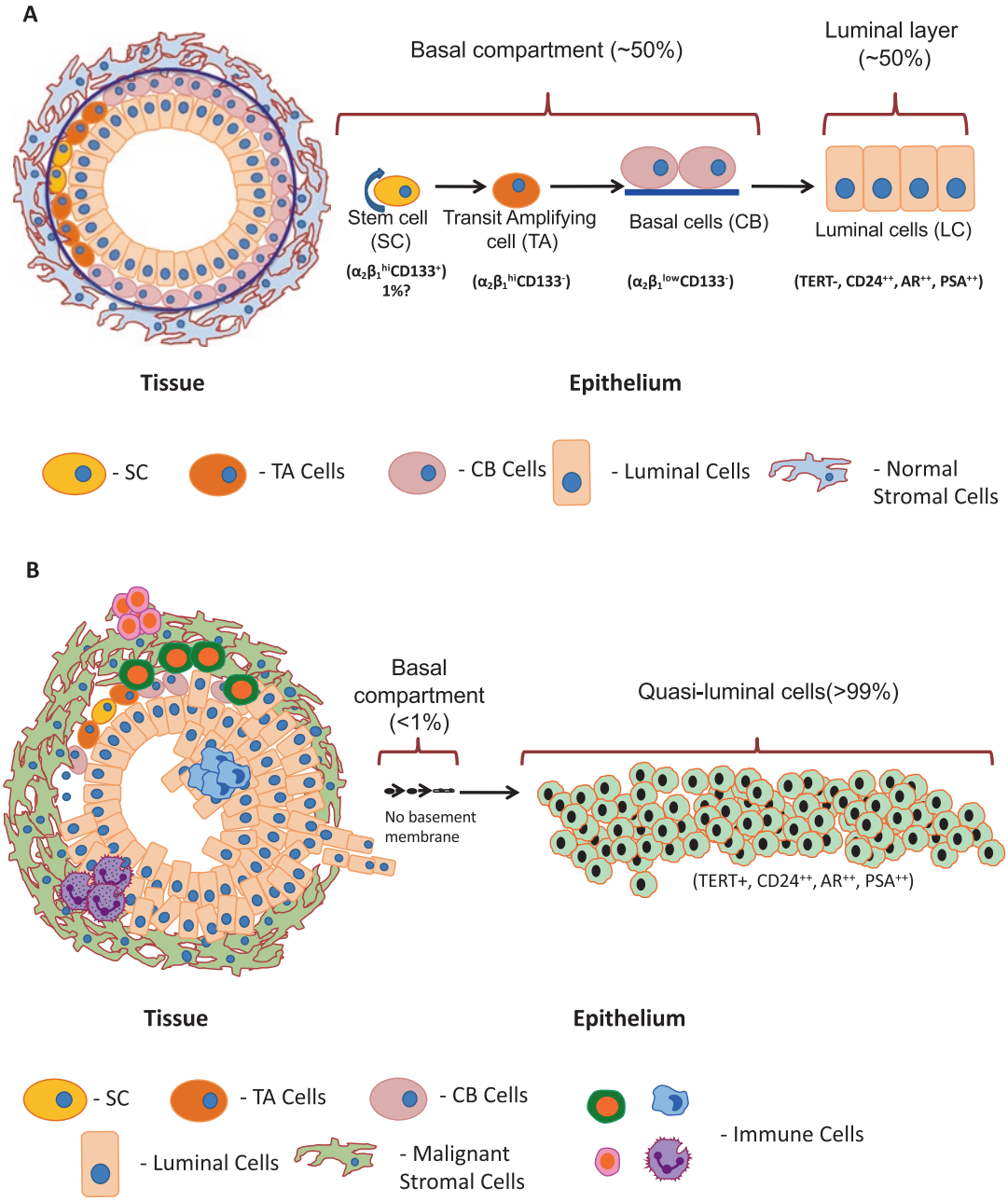


Fig. 1 Multicellular nature of normal and malignant human prostate tissues. (**a**) Multicellular nature of a normal prostate acinus. (**b**) Complex multicellular microenvironment of a malignant prostate gland

This hypothesis has resulted in the definition of carcinoma-associated fibroblasts (CAFs) as a discrete cell type which can be isolated from tumour areas. Such cells have a more stem cell-like phenotype, and are capable of inducing a carcinogenic phenotype in benign epithelial cultures from prostate such as BPH1, which usually form non-malignant cystic growths when grafted in combination with fibroblasts from normal areas of the prostate [15, 16]. The mechanism by which the benign cultures become frankly malignant in xenografts is likely to be complex, but there is good evidence to suggest that the TGFbeta signalling axis plays a key role [17].

Tracking Heterogeneity in a Low Mutation Tumour Such as Prostatic Adenocarcinoma

Traditionally cancer has been considered as a process driven by mutation in critical genes, and an increasing frequency of mutation detection as the tumour develops, including the development of resistance to multiple therapies. This certainly seems to be the case with a number of common tumour types such as small cell lung cancer [18], but prostate cancer belongs to a low mutation group [19] (illustrated in Fig. 2). Recent next generation sequencing (NGS) of whole prostate tumour biopsy DNA has confirmed this, while confirming the dogma that later stage cancers (after treatment failures) carry a higher mutagenic burden [20–22] compared to treatment-naïve tumours. One paradox in this data is the relative prevalence of both IDH1 and TMPRSS2-ERG lesions in early versus late cancers [22]. If the latter develops from the former, then the mutation states should be preserved, as shown in recent genomic sequencing studies of multiply and sequentially biopsied tumours [23, 24]. This is not the case, particularly as IDH1 mutations are undetectable in relapsed cancers [22], which argues either for (1) independent clonal origins for the relapses, probably not correct as specific mutations are shared with the primary tumour, or (2) that IDH1 mutation confers a transient advantage to the early stage cancers, and that the relapse originates in a less mutated progenitor cell, which makes up a small proportion of the original tumour mass.

In terms of cancer cell survival, mutation can be considered as a poor evolutionary step, particularly in a changing microenvironment manipulated by successive treatments. Whilst such non-reversible changes are observed in genes such as androgen receptor (AR) after therapy to block hormone responses in prostate cancers [25], there is increasing evidence that several tumour types achieve treatment resistance and the evolution of a fatal metastatic phenotype by means of epigenetic rather than mutagenic changes. A good recent example is the paucity of 'metastatic' mutations in malignant pancreatic adenocarcinoma, where the tumour evolution is apparently mediated by genomic methylation changes [26].

Cell Fate Decisions in Multicellular Prostate Tissues

Most studies have reflected on the 'oncogenic changes' required to produce the loss of growth and positional control which characterise human

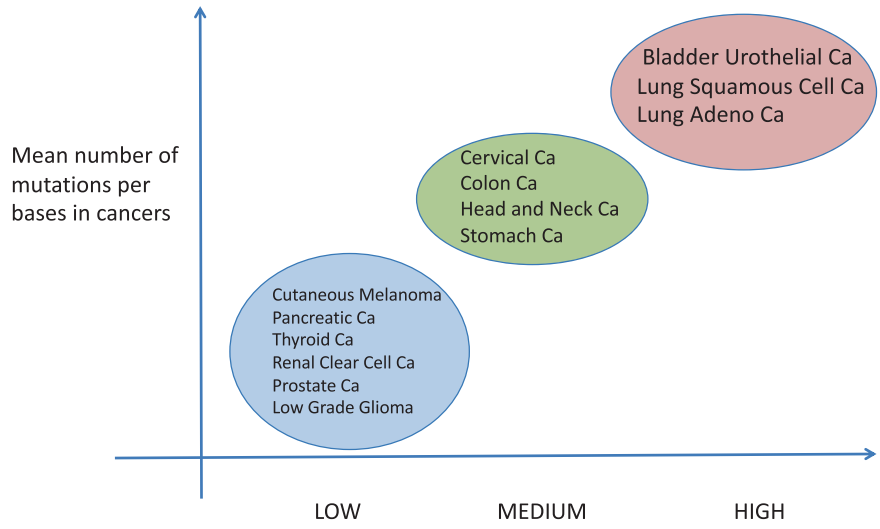


Fig. 2 Prostate cancers contain fewer biologically active mutations than many other common human tumours

cancers, and frequently used available cell lines [27] to define these changes and their downstream effects. As illustrated in Fig. 1, a cancer in man is a complex community of different cell types which can interact with one another in positive and negative ways. However, it is possible to generalise about cellular changes in prostate cancers as shown in Table 1.

When studied at gene expression or genomic level using mixed cell biopsies of tissues, contributions from minor populations are masked, and gene expression from the most transcriptionally active cells (secretory luminal cells), which can be up to three logs higher than in more quiescent cells, dominates most gene expression signatures. Of course, each biopsy (and patient) has variable proportions of the constituent cells which simply produces 'noise' in the final analyses and reduced statistical significance for the various markers. A good example of this was the TCGA attempt using a combination of mutations, DNA methylation (at promoters), copy number alterations, mRNA expression (including gene fusions), microRNA expression and protein expression to cluster mixed cell populations from 333 primary prostate cancers, which left about 26% of tumours unclassifiable even into seven subtypes [22]. Similarly, the expression signature of highly secretory immune cells also contaminates many 'tumour signatures' in blood DNA classifications [28], whilst stromal cell content provides the best indicator of malignancy in many colon cancer biopsy studies [29]. Such empirical findings may be useful in diagnosis, but the lack of a tumour cell component does not help in our *understanding* of key carcinogenic changes.

Table 1 Some essential characteristics of human prostate cancers

	Tumour property (primary prostate cancers)
1	Primary prostate cancers largely consist of replicating cells with an aberrant luminal phenotype (AR+, PSA secreting, changed energy requirements), whereas in normal tissues luminal cells rarely divide
2	Primary cancers have lost more than 99% of basal cells compared to normal prostate, where there is (in man) a contiguous basal layer lining prostatic glands
3	Basement membrane, which provides a barrier between the stromal and epithelial compartments, has all but disappeared in higher grade primary cancers
4	The stromal compartment in cancers has a novel more embryonal phenotype (see above) termed 'reactive stroma'
5	The intratumoural immune cell components, which include both lymphocytes and macrophages, undergo a distinct phenotypic switch

The one certainty in such analyses of gene expression, when cancer biopsies are compared to normal, is the predominance of luminal cell markers—since there are extremely small numbers of basal cells in prostate cancers. Indeed, the loss of expression of the strongly basal cell marker TP63 is a commonly used diagnostic aid to distinguish benign from malignant prostate disease [30].

The heterogeneity is also frequently ignored when the origins of castration-resistant prostate cancers and the ultimately fatal neuroendocrine form of the disease [31] are posited. In a homogeneous tumour model, there is a requirement for trans-differentiation of the luminal cancer cells into CRPC and NE tumours, which can be readily achieved by selection and treatment of cell lines such as LNCaP [32, 33]. However, the molecular tags of the relatively rare mutations in prostate cancer tells another story. Since it is unlikely that cancers can repair or lose mutations, why as discussed earlier do some mutations in early treatment-naïve cancers fail to make it into the CRPC form in the same cancers? There are a number of mutations (trunk or founder mutations) which are conserved—indicative of a common cellular origin. We have proposed that all prostate cancers contain a population of largely quiescent stem-like or progenitor cells which are resistant to most treatments and provide a mutated reservoir for the emergence of new cancer clones when the post-treatment microenvironment selects for a new variant cell type [34] (Fig. 3). Since many of the experiments are carried out in vitro, it has been argued that this is a cell culture artefact. However in man, an EORTC study [35] from 12 years ago provided strong evidence that the cells which regrow after androgen ablation are pre-existing in the tumour population, by comparing relapse rates in two cohorts of patients, one given imme-

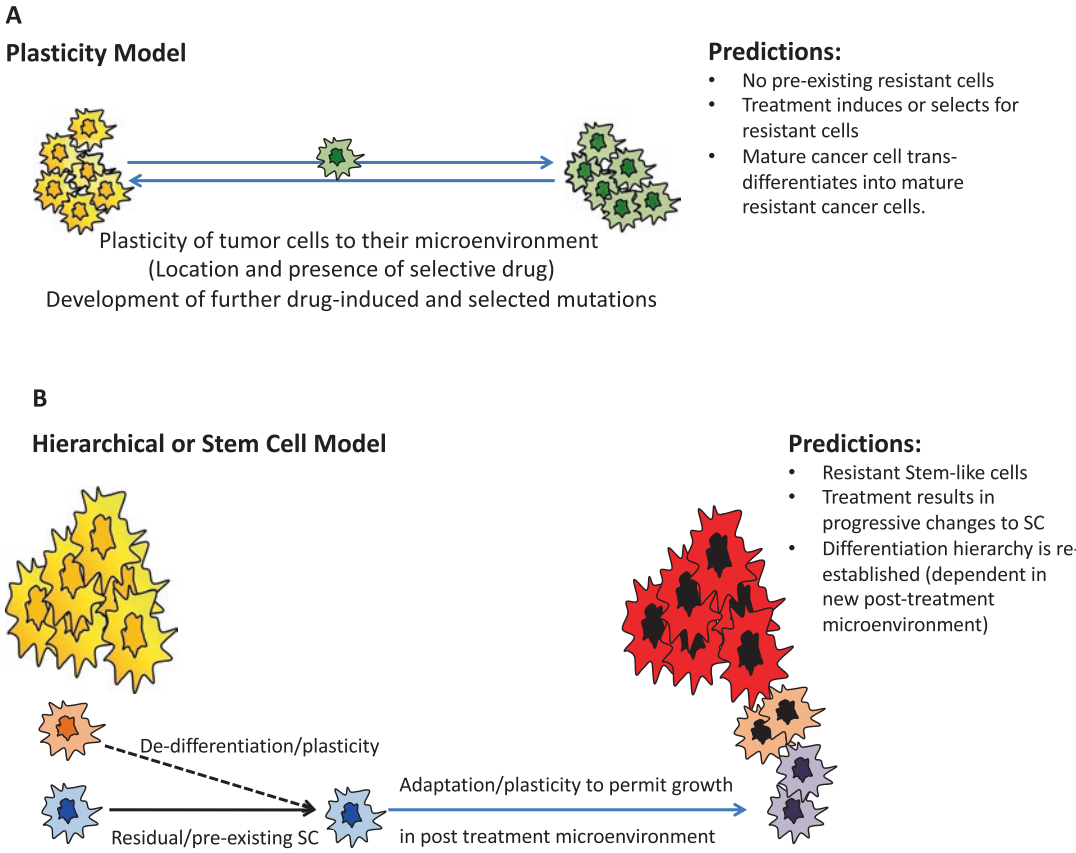
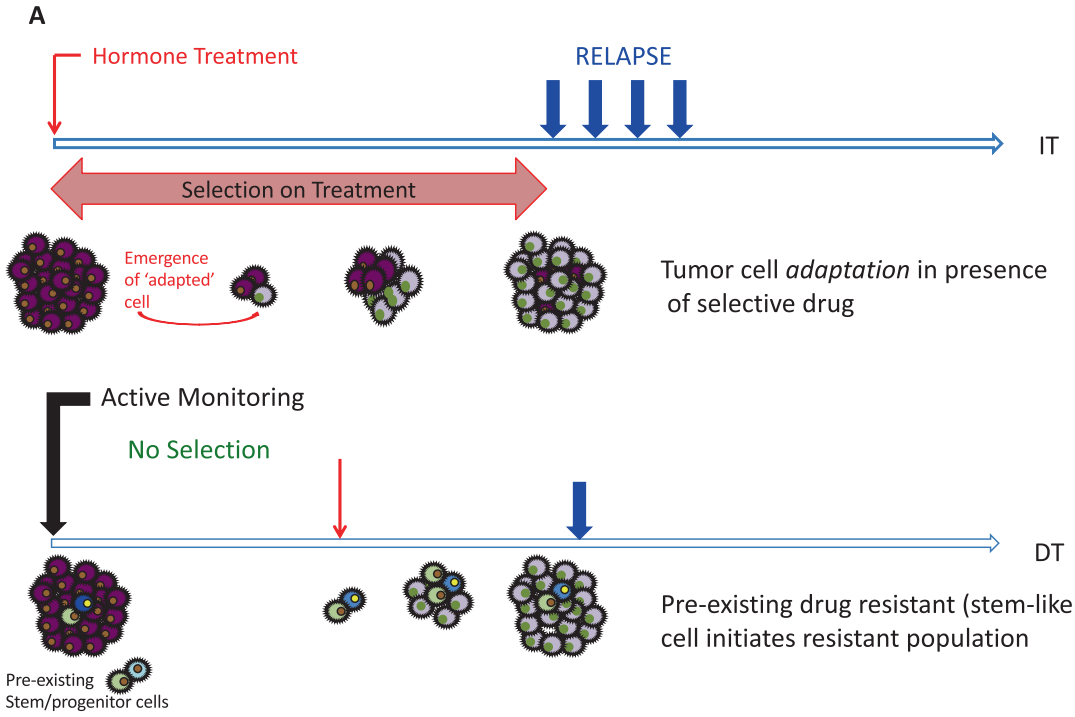


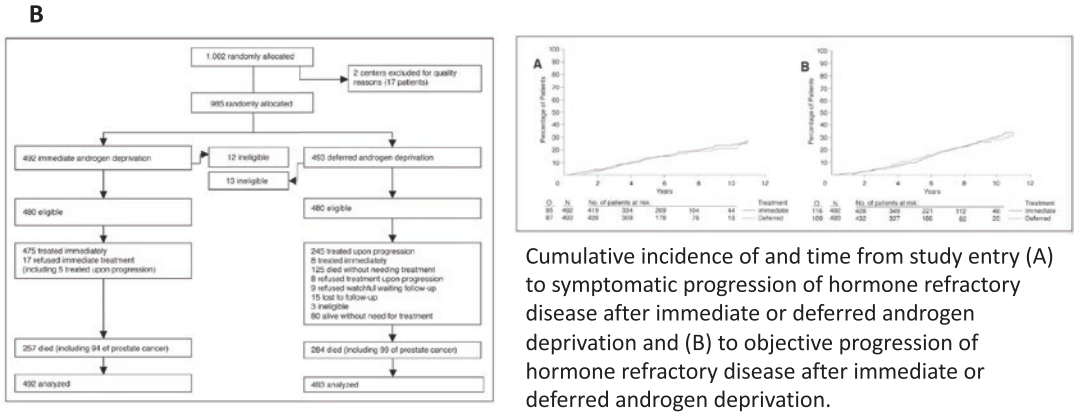
Fig. 3 Origins of tumour relapses in prostate cancers. Models are based on trans-differentiation (**a**) or regeneration from a common cancer stem/progenitor cell (**b**)

diate anti-androgen receptor drugs, and a second where the treatment was delayed until tumour progression was seen. Realistically, if the treatment is inducing luminal cancer cells to trans-differentiate, then the treated cohort would show higher and faster relapse. This was not observed in the trial—as illustrated in Fig. 4.

Small Non-coding RNAs as Controlling Factors of Gene Expression in Prostate Epithelial Differentiation?

To establish the factors which determine cell phenotype in the prostate, we have developed a fractionation procedure for prostate tissues [36], which enables both purification and ultimate culture of all cell types, with the exception of culturing both normal and cancer luminal cells. To identify the genes whose expression changes marked the epithelial differentiation process, we employed a pairwise analysis of total gene expression patterns, focusing on benign rather than malignant samples, since the benign epithelium had more consistent expression profiles [37]. We reasoned that genes with a regulatory role would change expression in a regular manner as clusters. The published data [38] revealed that genes showing a co-ordinate regulation during differentiation formed four distinct and non-overlapping sets (Fig. 5). Overall the regulatory networks with the highest significance in the transition from the most primitive stem-like epithelium to basal cells committed to differentiation into luminal cells were generic 'tissue developmental events', but more specifically retinoic acid and ROCK2 signalling. Upon the termi-





Cumulative incidence of and time from study entry (A) to symptomatic progression of hormone refractory disease after immediate or deferred androgen deprivation and (B) to objective progression of hormone refractory disease after immediate or deferred androgen deprivation.

Fig. 4 Similar relapse rates for prostate cancers after immediate and deferred androgen blockade. (**a**) Shows a selection model where mutations in the AR are induced or highly selected compared to a re-differentiation model from a stem-like progenitor. Original trial data showing identical relapse rates (**b**) from Studer et al. [35]

nal differentiation to luminal cells, all gene expression patterns are dominated by androgen signaling. But what controls the switch from the basal compartment (where retinoic acid signalling predominates) to luminal cells? A clue might come from the regulation of prostatic transglutaminase (hPTG), which belongs to one of the distinct four transcriptional co-regulation groups [39]. In the upstream control sequences of hPTG, as in many other related genes scattered throughout the genome, the binding sites for the retinoic acid receptor (RAR/RXR) are in close apposition on the genome with those for AR. However, the RAR/RXR sites are constitutively occupied in

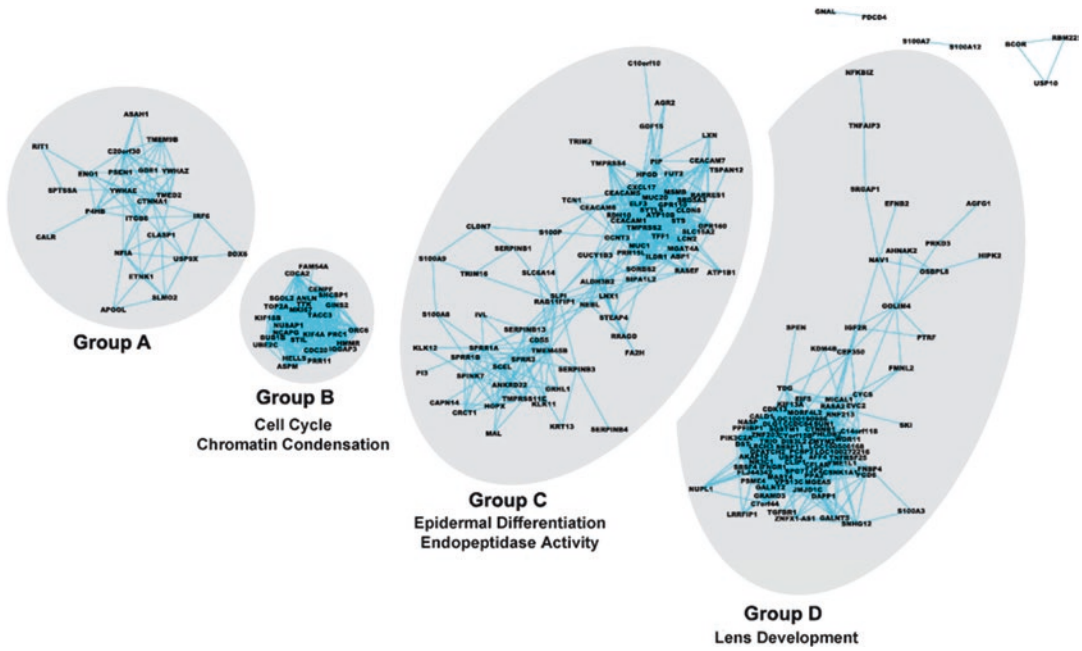


Fig. 5 Four non-overlapping sets of genes are co-expressed during differentiation of prostate epithelial cells. Data from Rane et al. [38]

the more committed basal cells, but not in the stem cell compartment. In contrast, AR must bind ligand in the cytoplasm before translocating into the nucleus to occupy its binding sites. The relative proportion of occupied binding sites defines the cell fate decision. The provision of activated ligand from retinol for RAR/RXR is likely to come as a paracrine interaction from the stem/progenitor cells which constitutively express both retinol and aldehyde dehydrogenases, but very low receptor levels. These AR-RAR dually regulated genes mark the transition but do not effect it—a function assigned for example to numerous transcription factors, such as a master controller like MYOD in muscle development [40].

But how is the simultaneous activation of transcription factors achieved in prostate? Hormones clearly provide part of the explanation, but in mammalian development, small non-coding RNAs play a central role [41]. To investigate this possibility, we analysed the expression patterns of microRNAs in the fractionated epithelial cell types. As shown in Fig. 6, the highest expression levels were seen in the least differentiated stem/progenitor cells, with a consistently progressive loss of expression towards the committed basal cells. When compared to other cell-type-specific miRNA expression patterns in online databases, the stem cell pattern most closely matched that from human embryonic stem cells [42].

However, reflecting the prostatic origin of the cells, the benign SC miRNA expression pattern also matched that of total miRNA in castration-resistant prostate cancers [42]. This agrees with a commonly observed SC-like mRNA gene expression pattern seen in CRPC [43]. But can any of these miRNAs act to control the differentiation-regulating transcription factors?

Many algorithms have been developed to identify the genes whose expression is modulated by a specific miRNA. However, these are based on the theoretical presence of miRNA recognition sites within the genome. The application of this analysis will confirm a miRNA target gene, but takes no account of whether the gene in question is expressed either in the target tissue or indeed the cell type of interest within that tissue. A classical example of ‘phantom’ gene expression control (by miRNA 143/145) was demon-

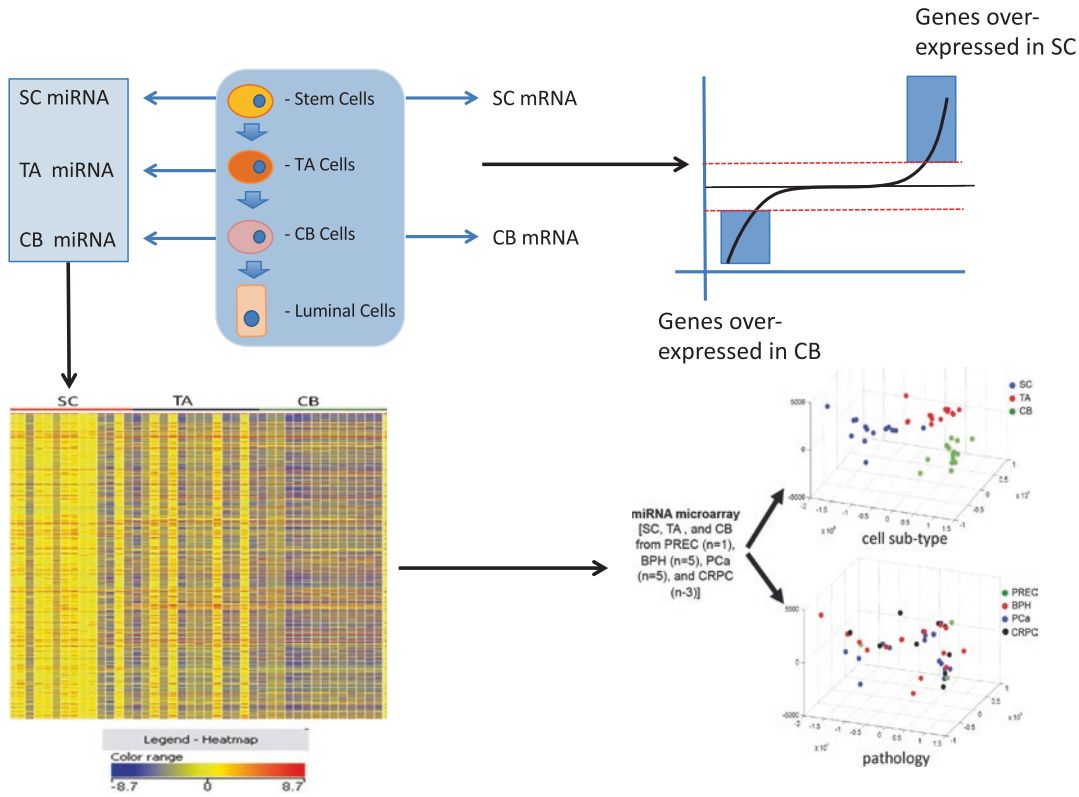


Fig. 6 Patterns of miRNA expression in fractionated cells from multiple prostate biopsies. Note the overall high levels of miRNA in the SC compartments where the expression profile is most closely related to that found in human embryonic stem cells and total CRPC miRNA patterns of expression. In keeping with the differentiation-linked functions of miRNAs, a principal component analysis was only able to align miRNA content with epithelial cell type, and not prostate pathology (normal, benign or malignant)

strated in mouse colon [44]. To address this, a specialised algorithm, which related *actual* mRNA expression in the prostate epithelium to the miRNA expression patterns, was applied [45]. This strategy identified miRNA 548-3p as a master controller of the transcription factors implicated in our earlier studies (RXR, VDR, GR, TAZ, SRF, HSF1 from [42]). miRNA 548-3p was consistently overexpressed in the stem-progenitor population but not expressed in the more committed basal cells. When expression was engineered in the committed basal cells, the result was an increase in the stem-like population in cell cultures, based both on increased colony forming (biological) and the upregulated expression of a set of stem/progenitor genes such as CD49b and f (integrins alpha 2 and 6, respectively) [42].

During development of the expression algorithm, the data was also aligned according to gene ontology functions in the differentiating epithelium. The most significant term was DNA repair/radiation resistance, with miR99a/100 as the most relevant miRNA. Previous studies had shown that the radiosensitivity of prostate cell lines (DU145 > PC3 > 22RV1 > LNCaP) was inversely related to the expression of miR99a/100. We had also demonstrated that the stem-like population displayed a markedly higher resistance to ionising radiation [38], and showed that this was a consequence of the highly condensed nature of the chromatin in the SC. The induction of increased stemness by introduction of miR548-3p into CB cells also resulted in an increased radiation resistance in the population. This aligned well with online data, which associated

endogenous miR548-3p expression in patients' tumours with a poorer prognosis for prostate cancer patients [42].

In a similar manner, when the lower expression of miR99a/100 in SC was engineered by introduction of miR inhibitors into the CB populations and various established prostate cell lines, an increased cell survival, assayed by colony forming activity, was observed. In this case, there was no accompanying change in the differentiated state of the cells (as seen with miR548-3p) assayed by a lack of expression of stem cell (NFkappaB, ID2, PROM1, SOX2) or EMT (VIM, CHDH1, FN1) markers. Systematic elimination of potential miR99a/100 target genes, based on our algorithm of prostate epithelial mRNA expression patterns, then identified two SMARC genes (A5 and D1), which had already been assigned a role in DNA damage repair, where their core function was to affect chromatin condensation levels, and to recruit BRCA1 and RAD51 DNA damage repair proteins to radiation-induced lesions [46]. In keeping with a cytoprotective property, upregulation of these SMARC genes was seen within 3 min of prostate epithelial cell irradiation.

There is also evidence that cytoprotection can be enhanced by glucocorticoids (GC), and when SMARC levels were quantified after dexamethasone treatment, they were also upregulated by GC. Whilst this provided a mechanistic proof, it also implies a potential controversy in the treatment of prostate cancer patients, where glucocorticoids are historically given to improve patient well-being. This implies that the addition of GC to a treatment protocol for radiotherapy would be likely to compromise the effectiveness of the treatment. To test this hypothesis, we used the GC response inhibitor mifepristone which resulted in an increased sensitivity to radiotherapy, whilst downregulating SMARC expression [46]. Thus an understanding of the basic biology of miRNA epigenetic control of cellular differentiation, using fractionated cells from patient biopsies, could ultimately influence prostate cancer treatment in patients.

Methylation of CpG Sites Distal from 'CpG Islands' as a Genomic Control of Differentiation and Carcinogenesis in the Prostate

As discussed earlier, prostate cancers have a particularly low rate of carcinogenic mutations. Recent studies of pancreatic cancers (which have an inherently higher mutagenic rate than PCa) showed that epigenetic changes such as differential genomic methylation could define the transition from organ confined to malignant cancers [26]. The ability of methylation-editing enzymes to change global methylation of CpG and other susceptible sites has also played a role in mammalian tissue differentiation [47]. In earlier comparisons of focused methylation within the promoters of target genes, we showed that the epigenetic modifications in established cell lines formed a distinct cluster, separate from that in primary cell cultures, and independent of pathology [48, 49].

To analyse cell-specific methylation patterns, freshly disaggregated tissues were fractionated as previously described to generate a basal fraction, a luminal cell fraction and stromal cells (as shown in Fig. 8a). Using the relatively conserved tissues from BPH [37], rather than cancers, we had already shown that even in primary cultures, distinctive and functional changes in gene expression were induced in vitro (Fig. 7b). However, such changes were limited, compared to those seen in a total gene expression comparison between multiple primary epithelial cell cultures, and benign (BPH1) or malignant (PC3) prostate epithelial cell lines (Fig. 7c).

After DNA purification from homogeneous cell fractions (of <1000 cells), assayed by both RT-PCR and FACS for cancer and differentiation markers (see Table 2), each DNA extract was divided, and one half was subjected to bisulphite conversion before sequencing of both cell populations [50].

Hierarchical clustering analysis was carried out on four matched human normal:cancer (defined as a patient with a majority of Gleason Grade 4 pathology in the biopsy) pairs of samples

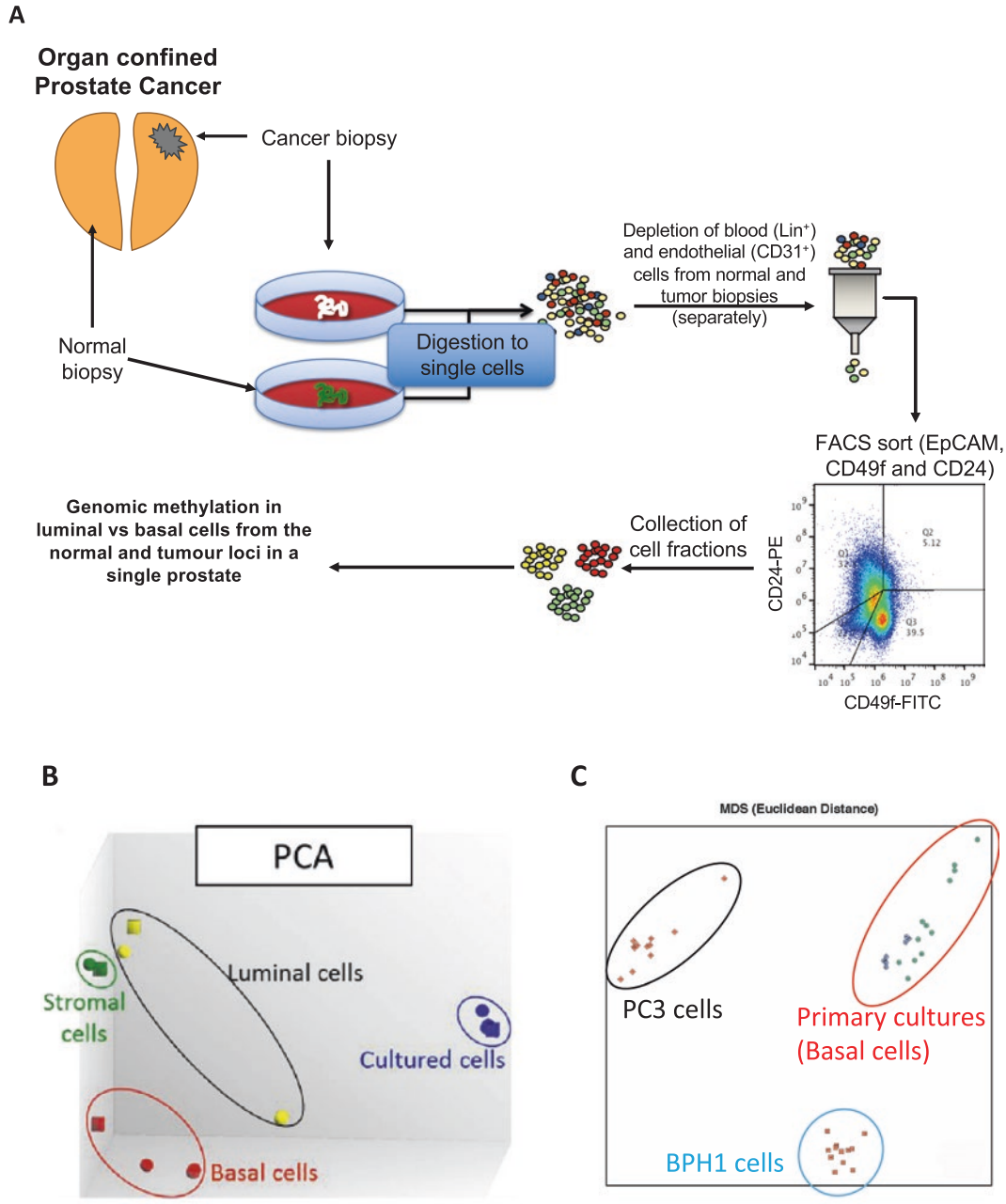


Fig. 7 Fractionation strategy for primary human prostate tissues. Multiple biopsy strategy and fractionation from radical prostatectomy tissues. (**a**) Gene expression changes (**b**) induced in benign prostate epithelial cell cultures, relative to tissues—data from Rane et al. [42]. (**c**) Gene expression differences between cell lines and primary cell cultures of prostate epithelial cells (unpublished data from Leanne Archer)

(i.e. 6 cell populations resulting in 12 datasets per patient). Some samples from patient 4 were lost during processing and only those with a high purity were processed.

Initial hierarchical clustering of the patients based on their different CpG methylation profiles revealed an enhanced ability to distinguish between patients, regardless of pathology. In any

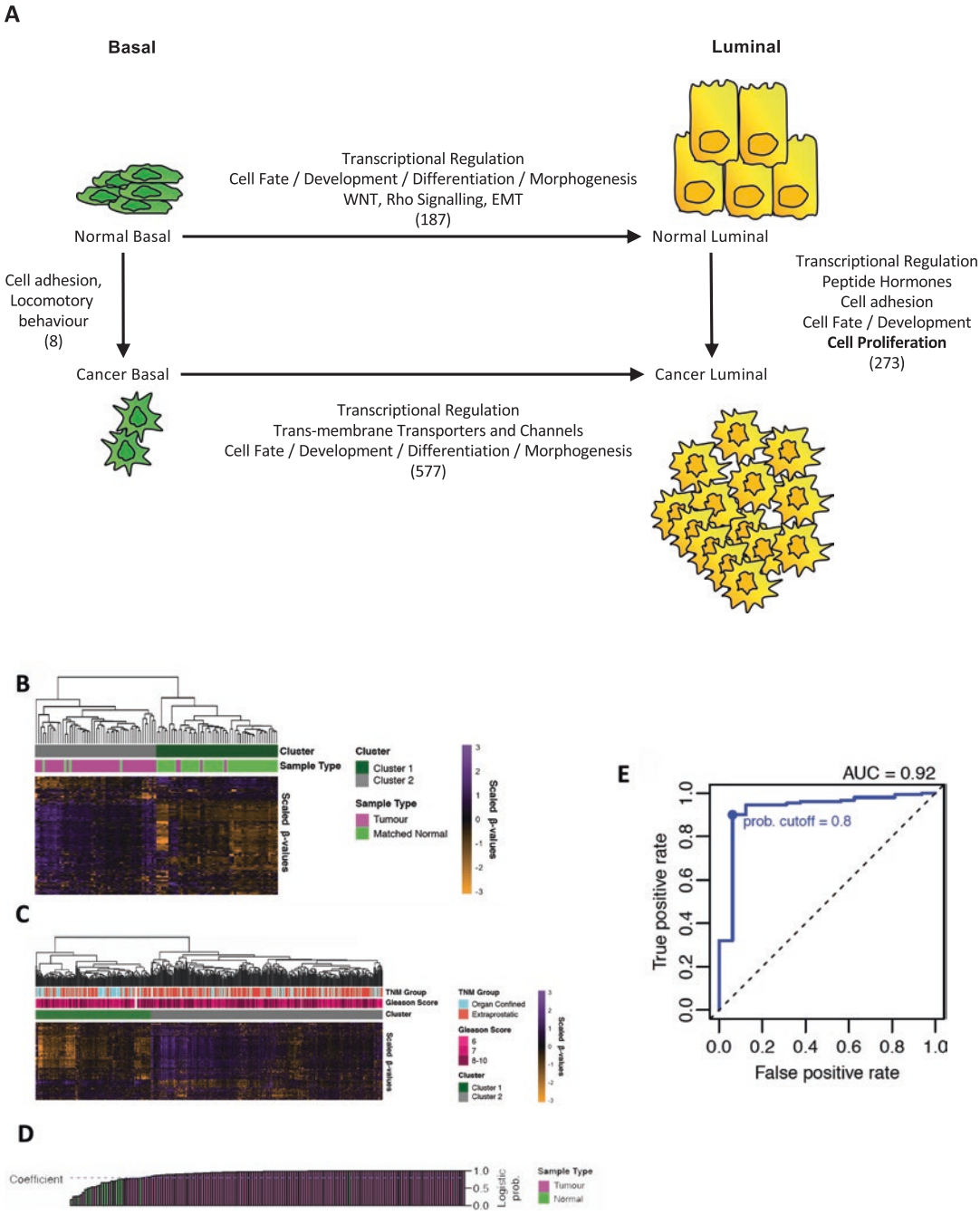


Fig. 8 Epigenetic segregation of normal and malignant tissues after cell fractionation to establish homogeneous patterns of genomic methylation. Gene ontology differences derived from CpG methylation measurements of fractionated cells from a single patient (**a**). Values for each transition refer to the number of DMRs which vary between each cell population. Clustering analysis of differentially methylated sequences using data from TCGA can distinguish cancer from normal cells with high efficiency (**b**). Application of the DMR data can also segregate malignant from organ-confined prostate cancers (TCGA data) independently of the gleason grade of the original cancer (**c**). (**d/e**) A 17 locus signature can be generated to distinguish normal from cancer tissues based solely on the pattern of DMRs. Data taken from Pellacani et al. [50]

Table 2 Selection and phenotype monitoring of cell fractions from fresh human prostate tissues

Cell type	Selection markers	Phenotype markers (RNA)	Phenotype markers (protein)
Normal basal cells	EPCAM+/CD24−/CD49f+	KRT5/14+; TP63+ ITGA6+/ECAM+	CK5/14++
Normal luminal cells	EPCAM+/CD24+/CD49f-	KRT8+/CD24+ EPCAM+	AR++/CK8+
Normal stromal cells	EPCAM−/CD24−/CD49f−	VIM+	VIM+/AR+
Cancer basal cells	EPCAM+/CD24−/CD49f+	AMACR+/KRT5/14+;TP63+/− ITGA6+/ECAM+	CK5+: CK14+
Cancer luminal cells	EPCAM+/CD24+/CD49f−	AMACR++/KRT8+; ECAM+	AR++, CK8+
Cancer stromal cells	EPCAM−/CD24−/CD49f−	VIM+	AR+

multipatient study therefore, this signature could mask the underlying pathogenic-related changes. Secondly, once individual signatures were removed, a strong cell-type-specific methylation pattern emerged. This could clearly distinguish stromal from both epithelial populations, where signatures were closely related. When the epithelial comparisons were made within each patient (an example of which is shown in Fig. 8a), linking the methylated CpG to adjacent genes and their functions, a number of gene ontology terms emerged as significant. Most changes were concerned with basal to luminal differentiation processes, which was the original aim of the study. For example, differentially methylated regions (DMRs) in a comparison of luminal and basal cells from normal prostate epithelium enriched for more than 500 terms. The hypermethylated set of gene ontology (GO) terms included many linked to prostate development or epithelial stem cell regulation. The hypomethylated DMRs were enriched for completely distinct GO terms principally related to androgen receptor signalling and responses to cytokines.

Unlike many previous studies which focussed on the previously determined 'CpG islands' clustered close to gene transcriptional start sites [51], we found that the most significantly altered sites were located at genomic locations more than half of which fell *outside* of known CpG islands, shores or shelves, and >70% were >5 kb distant from annotated transcriptional start sites. Thus, the differentially hypermethylated regions were enriched at loci previously defined as enhancers which were defined by three characteristic properties within the genomic sequence databases such as ENCODE: (1) evolutionary conservation of sequence and location relative to the gene whose expression is under enhancer control; (2) binding sites for known transcription factors defined by chromatin immunoprecipitation (ChIP); (3) the presence of 'open' chromatin delineated by DNAaseI hypersensitivity. The principal hypomethylated loci included extragenic repetitive sequences such as LINE and LTR, but not SINE repetitions. For example, hypermethylated enhancers were highly enriched for TFBSs of *TP63*, *TP53* and *NF1*, and hypomethylated DMRs for *FOXA1*, *p65-NFkB* and *GATA3*. A reassuring differential methylation was also seen for two well-established 'epigenetically controlled' genes GSTP1 and CCDC8, although this was characteristic of a basal to luminal change rather than a cancer-specific hypermethylation, within 5 kB of the published transcriptional start sites, i.e. aligned as before with promoters rather than enhancers [52].

It was clear from the data that, at the level of CpG methylation, there were relatively few cancer-specific methylation changes between the basal cell compartments of normal and malignant tissues (Fig. 8a). However, in the luminal compartment, the differentially methylated enhancer sequences detected were enriched for those affecting the expression of genes from the PRC2 complex such as EZH2 and SUZ12, previously shown to be overexpressed in prostate cancer [53, 54], and the appropriate gene ontology terms such as

metabolic processes, epithelial development and most notably cell proliferation. This agrees with the major physiological change between normal and cancer luminal cells: normal luminal cells are terminally differentiated and rarely divide, whereas the luminal-like cells in prostate cancers are characterised by uncontrolled cell division.

Because the dataset included patient-matched normal:cancer samples, after further elimination of cell-type-specific changes, we were able to assess cancer-specific changes in the different cell phenotypes on a patient-by-patient basis. However, it should be noted that many of these were also present in a comparison of cancer luminal and cancer basal cells (differentiation)—the cancer significance was emphasised by the absence of such changes in a normal luminal–normal basal comparison. Since luminal cells were the principal constituent of the cancer epithelial populations, we next sought to identify cancer-specific methylation sites in comparisons of normal and malignant luminal cells. The elimination of the approximately 50% basal cell content from the 'normal' samples ensured that we did not rediscover any new upregulated AR stimulated luminal markers in these comparisons.

Since our original discovery sample number was low [50], we sought to apply the cancer luminal signature to a larger number of cancers from The Cancer Genome Atlas (TCGA) database which contains 50 PCa samples with matched normal counterparts, 452 additional PCa samples without normal counterparts and 1 metastatic PCa sample. Unfortunately the TCGA data was generated with older array technology, so the sequence data was converted to 100 bp bins, to align the two data sources. In the TCGA database, 255 array probes overlapped the 1472 DMRs we showed by sequencing to be differentially methylated in the cancer luminal to normal luminal comparison. When used to analyse the matched pairs in TCGA, the differentially methylated regions distinguished 50 cancers from matched patient normal tissues with close to 100% efficiency (TPR = 0.92, TNR = 0.92, Chi-squared test p-value = 2.4×10^{-16}) as shown in Fig. 8b (taken from [50]).

Using this reasoning, since we had eliminated normal elements, the same analysis was carried out with all 553 cancer samples in the TCGA dataset, resulting in a similar outcome, with one cluster highly enriched in normal samples (Chi-squared test p-value = 1.7×10^{-39}). Intriguingly, this clustering also appeared to divide the PCa samples into two main groups, according to their CpG methylation differences from the normal samples. Exclusive analysis of the cancer samples confirmed this clustering pattern and showed that one cluster was significantly enriched for samples with extra-prostatic extensions (pT3 or pT4 in the TNM classification, with a Chi-squared test p-value <0.005) in the absence of any significant differences in Gleason score (Chi-squared test p-value >0.1) (Fig. 8c).

The requirement to use an extensive panel of differentially methylated regions (DMRs) to achieve classification is probably an indicator of the multifactorial and diverse mechanisms required to achieve cancer cell malignancy. However, using as few as 17 DMRs we were able to differentiate cancer from normal luminal cell in data from mixed cell biopsies in TCGA with a 92% effectiveness (Fig. 8d, e), indicating that further more selective analysis could result in an epigenetic differentiation of the elusive 'tiger' prostate cancers which require immediate aggressive treatment from the 'pussycats', which are best treated by continuous monitoring rather than invasive oncological procedures. Since this was achieved in routine biopsies of intra-prostatic early stage disease, it implies that the fate of a prostate cancer is programmed into its genome, not by mutation but by epigenetic means at a relatively early stage, and that 'grade progression' to a more malignant state may exist [55], but is probably relatively rare [56].

Is Epigenetic Change the Smart Reaction to Changing Microenvironments in Prostate Cancers?

Our primary aims in this research were to dissect potential epigenetic mechanisms of gene expression control, and to distinguish the changes related to cellular differentiation, from those with a mechanistic importance in carcinogenic change.

In contrast to most other studies, our target materials were cells taken directly from prostate cancer patients, employing the ultimate control: cells of the same lineages from normal regions of the same patients' prostates. We had previously shown a distinct difference in the histone modification profiles, related to chromatin condensation in different cell populations, which could mark more undifferentiated cells. However, these chromatin marks were largely independent of the pathology in the prostate. On a primary screen, the same was largely true for global changes in miRNA expression: which were first identified as differentiation-linked epigenetic controls [57]. However, as shown above, a number of cancer-specific changes were related to DNA damage and repair by extracting actual gene expression levels, and matching these to the inverse of changes in miRNA in the same cell populations. Both the ability of miR534 (overexpressed in the prostate SC population) to preserve a stem-like state which was resistant to radiation treatment and the radio-protective activity of low levels of miR99a/100 via the activation of SMARCs provide a rationale for the survival of a stem-like population after patient radiotherapy. The deleterious effects of glucocorticoid administration, which is clinically acceptable, may have to be reconsidered based on their ability to similarly activate SMARCs.

The overall higher levels of miRNA expression and the existence of 'poised' or bivalent chromatin in the stem-like cells argue for an epithelial population within both normal and cancer populations which is capable of rapid reaction to a change in microenvironment, after cancer treatment for example. The emergence of a stem-like miRNA signature in the CD133+/a2b1 high/CD44+ population, based on homology with not only human embryonic stem cells but also and surprisingly CRPC tissues, indicates that advanced prostate cancers retain this flexibility, which can only be enhanced by the increased frequency of mutations in DNA damage repair genes in CRPC. The epigenetic ability to rapidly switch phenotype to a treatment resistant cell type, which permits establishment of permanent resistance after subsequent mutation and selection, is entirely consistent with the development of drug resistance in advanced prostate cancers. Since more cells contain the stem-like miRNA pattern in the most advanced cancers, then the possibilities for resistant development are increased as the tumour successively escapes from treatments. This would be consistent with a trans-differentiation model in CRPC drug resistance, whereas the lower content of stem-like cells in treatment-naïve tumours would argue for a stem/progenitor origin of the resistant population.

Conclusion

Prostate cancers are multicellular and complex in composition. In scientific attempts to simplify analysis, we have adopted a reductionist approach, using established and well-characterised cell lines. It is clear that with current comprehensive gene expression analyses, these basic cell line tools, established for more than 40 years in the laboratory, no longer represent the detail in the actual tumours. The more we push their relevance to drug response and tumourigenesis, the more apparent this becomes, i.e. apart from well-characterised hormone responses, translation from lab to bedside has been only moderately successful, and has not extended lifespans for more than a few months in most cases.

In fact, the mere act of primary culture induces epigenetic changes which alter the expression of key genes, even in benign prostate tissues, which show much less inter-patient variability compared to cancers [37]. Recent attempts to classify prostate cancers according to clinical outcome on the basis of both genomic and transcriptome changes have disappointed, even when biopsies of tumour tissues were available. For example, the most comprehensive TCGA survey [22] between a quarter and a third of all cancers was 'unclassified' even after multiparametric analysis.

The data we have presented in this article argues that there is an inherent 'patient-specific' pattern of variation, shown here at the epigenetic CpG methylation level. On top of this there is the

cellular composition variation between tumours, not only at the level of epithelial cell types but also the degree of stromal cell and immune cell content between patients. This cannot be truly represented, even in mouse models of the disease, unless we can break heterogeneity, by subfractionating fresh tumours into their component parts. Since most TCGA data has been generated from mixed cell populations, there is now a strong argument in favour of adopting a single cell (or small homogeneous cell number) approach to all new tumour cell analyses, in order to make genomics truly relevant for clinical application.

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Small Molecule Inhibition of Glycogen Synthase Kinase-3 in Cancer Immunotherapy

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Abstract

Immune checkpoint blockade (ICB) has proved successful in the immunotherapeutic treatment of various human cancers. Despite its success, most patients are still not cured while immunogenic cold cancers are still poorly responsive. There is a need for novel clinical interventions in immunotherapy, either alone or in conjunction with ICB. Here, we outline our recent discovery that the intracellular signaling kinase glycogen synthase kinase-3 (GSK-3) is a central regulator of PD-1 in T-cells. We demonstrate the application of small molecule inhibitor (SMI) approaches to down-regulate PD-1 in tumor immunotherapy. GSK-3 SMIs were found as effective as anti-PD-1 in the elimination of melanoma in mouse models. We propose the development of novel SMIs to target co-receptors for the future of immunotherapy.

Keywords

T-cells · Glycogen synthase kinase-3 · Immunotherapy · PD-1

Introduction

The past years has seen exciting progress in the application of "immune checkpoint inhibitors" (ICI) to treat various human cancers [1–3]. Monoclonal antibodies (mAbs) are used to block the engagement of negative co-receptors such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with their natural ligands. Despite this success, most patients are not cured while certain weakly immunogenic cancers are poorly responsive. The present shortage of actionable therapeutic targets and the poor prognosis for patients highlight a need for evaluating novel clinical interventions.

The co-receptor programmed cell death 1 (PD-1; also known as PDCD1) is a member of the B7 gene family that negatively regulates T-cell function [4–6]. The co-receptor binds to ligands, programmed cell death ligand 1 and 2 (PD-L1/L2) which is expressed on lymphoid and non-lymphoid cells [7–9]. PD-1 is an activation antigen and contributes to the exhaustion of $CD8^+$ T-cells during chronic infections, autoimmunity, and cancer [10, 11]. In this context, immune checkpoint blockade with anti-PD-1 or anti-PD-L1 has proven successful in the treatment of

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human cancers, either alone or in combination with anti-CTLA-4 [12, 13]. The blockade of PD-1 or PD-L1 restores T-cell functionality in various in vivo models [14], while PD-1 expression on tumor-infiltrating $CD8^+$ T-cells correlates with impaired effector cell function [5, 15]. PD-L1 expression on tumors can facilitate escape from the host immune system [6] and can serve as a prognostic factor [16].

The nature of the intracellular signaling pathways that regulate PD-1 expression on T-cells has been the subject of much interest. *Pdcd1* expression can be positively and negatively regulated by different transcription factors such as nuclear factor of activated T-cells (NFAT), Forkhead box protein O1 (FoxO1), Notch, activator protein 1 (AP1), and Blimp1 (B-lymphocyte maturation protein 1) [17–20]. Despite this, the identity of the upstream signaling event(s) that control PD-1 expression has been unclear. We and others previously showed that T-cells are activated by protein-tyrosine kinases $p56^{lck}$ and ZAP-70 [21, 22]. $p56^{lck}$ binds to co-receptors CD4 and CD8 [23–25] and phosphorylates immune receptor activation motifs (ITAMs) needed for ZAP-70 recruitment to the TCR-CD3 complex [21, 24, 26]. By contrast, glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that is active in resting T-cells and becomes inactivated with T-cell activation [27, 28]. Differentially regulated isoforms of GSK-3 α and β differ in their N- and C-terminal sequences and can influence pathways initiated by diverse stimuli. The inactivation of GSK-3 can be mediated by several upstream kinases including protein kinase B (PKB/AKT). In $CD4^+$ T-cells, GSK-3 promotes the exit of NFAT from the nucleus [29, 30]. TCR and CD28 ligation phosphorylates and inactivates GSK-3 [31–33], enhancing T-cell proliferation [31]. This engagement of PKB/AKT and GSK-3 in T-cells operates independently of the guanine nucleotide exchange factor VAV-1 [32]. Clinical trials using GSK-3 inhibitors have been undertaken in the treatment of type II diabetes and various neurological disorders [28, 34, 35].

Recently, we reported that the inactivation of GSK-3α/β with small interfering RNAs (siRNAs) and drug inhibitors specifically down-regulate PD-1 expression for enhanced $CD8^+$ CTL function and clearance of viral infections [36]. The approach has introduced the possibility that small molecule inhibitors (SMIs) of GSK-3 may be effective in the downregulation of PD-1 in the treatment of cancer. Here and in recent publications [37, 38], we show that SMIs of GSK-3 are as effective as anti-PD-1 in the control of B16 melanoma and EL4 lymphoma growth in mice. Our findings demonstrate the successful application of a GSK-3 inhibitor for the downregulation of PD-1 on T-cells in cancer immunotherapy.

Materials and Methods

Mice and Cells

C57BL/6 mice were used alongside OT-1 Tg mice. Spleen cells were treated with a hypotonic buffer with 0.15 M NH4CL, 10 mM KHCO3, and 0.1 mM EDTA, pH 7.2 to eliminate red blood cells before suspension in RPMI 1640 medium supplemented with 10% FCS, 50 μM beta-mercaptoethanol, sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and streptomycin (GIBCO). B16F10 melanoma and EL4 lymphoma cells were cultured in DMEM medium that was supplemented as above. All mouse experiments were approved by the Home Office UK (PPL No. 70/7544).

Antibodies/Reagents

The following antibodies were used in experiments: Anti-CD3 (2C11), anti-PD-1 (CD279, J43), anti-granzyme B, and anti-T-bet (Abcam plc); anti-GSK-3α/β, CD279 (clone EH12.2H7) coupled FITC and mouse IgG1 FITC control (Biolegend); conjugated antibodies, anti-CD8α (clone, 53–6.7), anti-CD4 (clone, RM4–5), CD44, CD62L, CD25, and CD69 (eBioscience); GSK3 inhibitors SB415286 3-(3-chloro-4-hydroxyphenylamino)-4-(2-nitrophenyl)-1H-pyrrole-2,5-dione and AZ1080 (Abcam plc); $OVA_{257–264}$ peptide (Bachem Ag).

Flow Cytometry

Flow cytometry of antibody staining of surface receptors was conducted by suspending 10^6 cells in 100 μL PBS and adding antibody (1:100) for 2 h at 4 °C. Cells were then washed twice in PBS and in some cases suspended in 100 μL PBS with secondary antibody for a further 1 h at 4 °C. Cell staining was analyzed on a BD FACS Calibur flow cytometer and by FlowJo software. For intracellular staining, cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.3% saponin (Sigma–Aldrich) and stained with the desired antibody in saponin-containing PBS for 2 h at 4 °C, followed by a secondary Ab incubation where primary antibodies were not conjugated.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Single-strand cDNA was synthesized with an RT-PCR kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the RNA polymerase chain reaction (PCR) core kit (Applied Biosystems). Quantitative real-time PCR used SYBR green technology (Roche) on cDNA generated from the reverse transcription of purified RNA. After preamplification (95 °C for 2 min), the PCRs were amplified for 40 cycles (95 °C for 15 s and 60 °C for 60 s) in a sequence detection system (PE Prism 7000; Perkin-Elmer Applied Biosystems). The exponential phase, linear phase, and plateau phase of PCR amplification were carefully monitored to ensure a measurement of real-time transcription (33). mRNA expression was normalized against GAPDH expression using the standard curve method. PD-1-FW, 5-CCGCCTTCTGTAATGGTTTGA-3; PD-1-RV, 5-GGGCAGCTGTATGATCTGGAA-3; T-bet-FW, 5-GATCGTCCTGCAGTCTCTCC-3; T-bet-RW, 5-AACTGTGTTCCCGAGGT GTC-3; GAPDH-FW, 5-CAACAGCAACTCCCACTC TTC-3; GAPDH-RW, 5-GGTCCAGGGTT TCTTACTCCTT-3.

Melanoma Lung Tumor Establishment in Wild Type Mice

B16 melanoma cells (2×10^5 taken from the log phase of in vitro growth, OVA peptide pulsed or non-pulsed) were transferred intravenously into syngeneic C57BL/6 male mice of 10–12 weeks old. The lungs were removed 14 days after the transfer, and visible metastatic colonies on the lungs were counted.

Isolation of Tumor Infiltrating Lymphocytes (TILs)

Solid tumors or nodules from lungs were harvested from mice at the time indicated. Tissue was disrupted using a blade and then incubated in HBSS solution containing 200 units/mL of collagenase at 37 °C for 2 h. Tissue was then passed through a strainer and cells collected and layered onto ficoll before centrifugation. Tumor infiltrating cells were then collected from the lymphocyte layer.

Statistical Analysis

The mean and SE of each treatment group were calculated for all experiments. The number of samples is indicated in the figure legends. Unpaired Student's *t* tests or ANOVA tests were performed using the InStat 3.0 software (GraphPad). In certain instances, statistics were done using 2-way ANOVA, or by non-parametric Mann Whitney at each time point. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

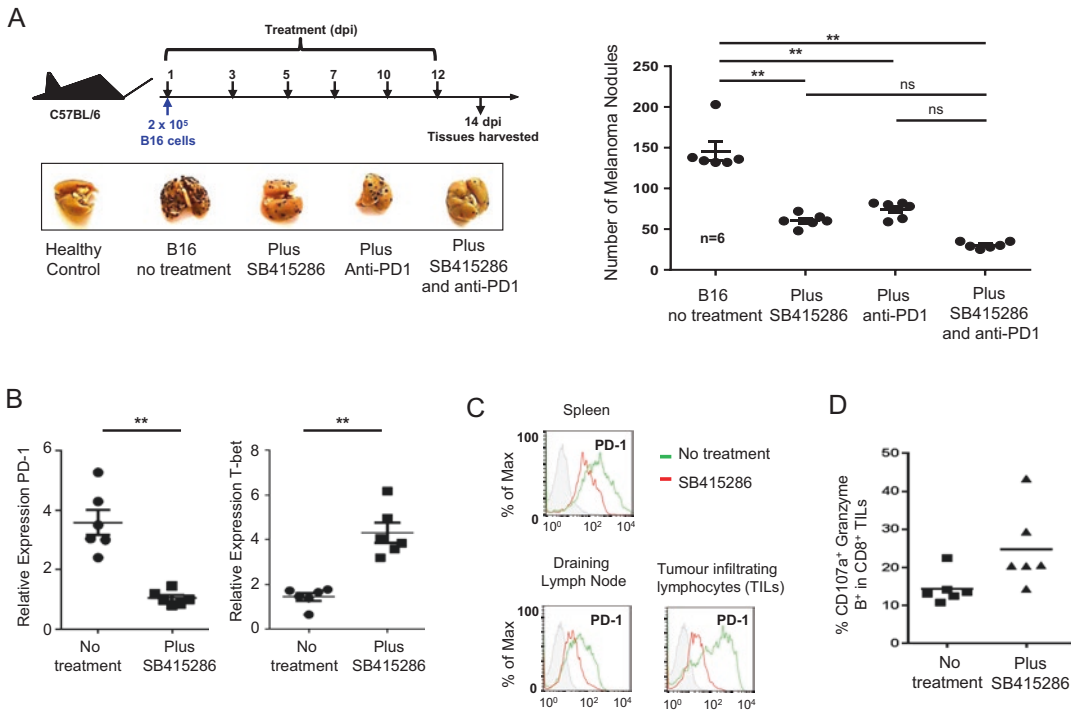

Fig. 1 GSK-3 inhibition, anti-PD-1, and combination therapy have identical effects in inhibiting pulmonary metastasis of B16 melanoma. (**a**) Schematic representation of treatment regime (left upper panel). Histogram showing the number of lung spots per animal with or without the described treatment (right panel), photograph below shows one example of each group ($n = 6$; number of mice: >24). (**b**) Quantitative real-time PCR of PD-1 and T-bet transcription of splenic T-cells from animals used in (**a**). (**c**) Flow cytometric profiles of PD-1 expression for T-cells isolated from spleen, draining lymph nodes (dLNs) and tumor infiltrating lymphocytes (TILs) (data representative of six samples). (**d**) % of $CD8^+$ tumor infiltrating cells expressing granzyme B as determined by flow cytometry ($n = 6$); $^{**}P < 0.001$

Results

GSK-3 Inhibits the Growth of Intravenous and Intradermal Injected Tumors

To assess whether the downregulation of PD-1 by GSK-3 inhibition was effective in limiting tumor growth, B16 tumor cells were injected intravenously into C57BL/6 mice with the GSK-3 inhibitor SB415286 and/or anti–PD-1 (Fig. 1a). The optimal dose of SB415286 and anti–PD-1 established in this model was 200 μg and 100 μg/injection/mouse, respectively. The SMI or antibody was administered following the regime depicted in Fig. 1a, followed by a harvest of lungs on day 14 and the numbers of B16 nodules assessed. Treatment witrh the GSK-3 inhibitor SB415286 reduced the number of B16 spots from a mean of 145–60 (i.e., >55% inhibition). This effect was comparable with anti–PD-1, which showed a mean of 70 spots (i.e., >50% inhibition). Furthermore, the combination of SB415286 and anti–PD-1 had the same effect as SB415286 and anti–PD-1 individually ($n = 6$). GSK-3 inhibition reduced *Pdcd1*(PD-1) transcription in T-cells from isolated spleen of tumor-bearing mice (i.e., 3.7–0.8), concurrent with an increase in *Tbx21* (T-bet) transcription (i.e., 1.6–4.2; Fig. 1b). Flow cytometry confirmed that a GSK-3 SMI reduces PD-1 expression on T-cells from the tumor [i.e., tumor infiltrating lymphocytes (TIL), spleen, and draining lymph nodes] (Fig. 1c). Concurrent with reduced tumor growth, SB415286 treatment increased the percent of $CD8^+$ TILs expressing $CD107a^+$ (Lamp1) and granzyme B (GZMB; i.e.,

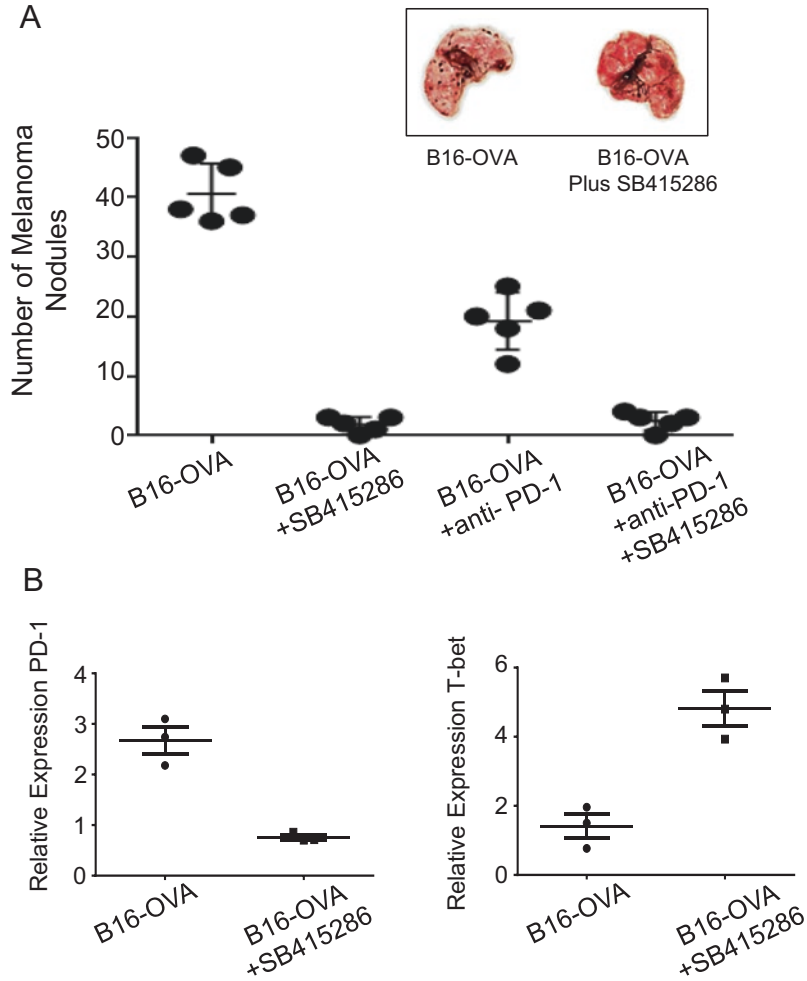

Fig. 2 Effects of GSK-3 inhibition and anti-PD-1 in a B16 lung model presenting OVA peptide. OT-1 Tg mice were injected with 2×10^5 B16-OVA intravenously, followed by intra-peritoneal injection of either PBS or SB415286, and lungs were analyzed on day 14. (**a**) Histogram showing the effects of SB415286, anti-PD-1, and combinations on the numbers of melanoma spots on lungs. Upper inset: photograph of an example of nodules on lungs. (**b**) qPCR analysis of extracted spleen T-cells confirmed a marked decrease in PD-1 expression (left panel) concurrent with increased T-bet (right panel)

mean % of 14–23), indicative of an increased presence of $CD8^+$ killer T-cells in the tumor mass (Fig. 1d). These data showed that the downregulation of PD-1 with a SMI of GSK-3 can be as effective as anti–PD-1 in the control of B16 pulmonary metastasis in mice.

In a related model, we compared the effects of GSK-3 inhibition versus anti-PD-1 on the presence of lung nodules using B16 cells presenting OVA peptide, in OT-1 Tg mice [39] (Fig. 2a). OT-1 Tg mice were injected with 2×10^5 B16-OVA intravenously, followed by intra-peritoneal injection of either PBS or SB415286, and lungs were analyzed on day 14. SB415286 injection resulted in a decrease in the number of B16 nodules from a mean of 42 for untreated controls to 1–4 spots in drug-treated mice (i.e., >90% reduction) (Fig. 2a; also see upper inset). Anti-PD-1 also reduced the number of nodules from 41 to a mean of 17 in treated mice. The combination of SB415286 and anti-PD-1 reduced the number of nodules to that seen using SB415286 alone. In this regime, GSK-3 inhibition appeared more effective than anti-PD-1 treatment. qPCR analysis

of extracted spleen T-cells confirmed a marked decrease in PD-1 expression concurrent with increased T-bet (right panels). These data showed that GSK-3 inhibition by SMIs can be as effective as anti-PD-1 in the control of B16 tumor cell growth.

To further assess whether GSK-3 inhibition elicited its effect primarily via its ability to inhibit PD-1 expression, we compared the ability of *GSK-3α/β* conditional knockout mice versus PD-1-deficient mice (*Pdcd1−/−)* to control B16 tumor growth. GSK-*3α/β* conditional knockout (*GSK-3α/β−/−*) mice were generated from GSK-3 alpha flox/flox/beta flox/flox mice crossed with Lck Cre^{+} mice resulting in mice in which T-cells were devoid of both GSK-3alpha and beta. *GSK-3α/β−/−* versus *Pdcd1−/−* mice showed the same control of the growth of B16 tumor cells (Fig. 3). Each mouse strain showed that same reduction in the number of B16 nodules as assessed on lungs (i.e., from 110 nodules to <10 in both sets of mice). Further, the injection of SB415286, or another GSK-3 inhibitor AZD1080, in *Pdcd1−/−* mice had no additional effect in reducing the number of nodules (Fig. 3a, upper inset). Likewise, the injection of anti-PD-1 had no further effect on the number of nodules in *GSK-3α/β−/−* mice (Fig. 3b, upper inset). These data show the efficacy of GSK-3 inhibition in the promotion of T-cell responses for the elimination of tumors.

Discussion

Immune checkpoint blockade with anti-PD-1 or anti-PD-L1 has proven to be a highly promising treatment of human cancers, either alone or in

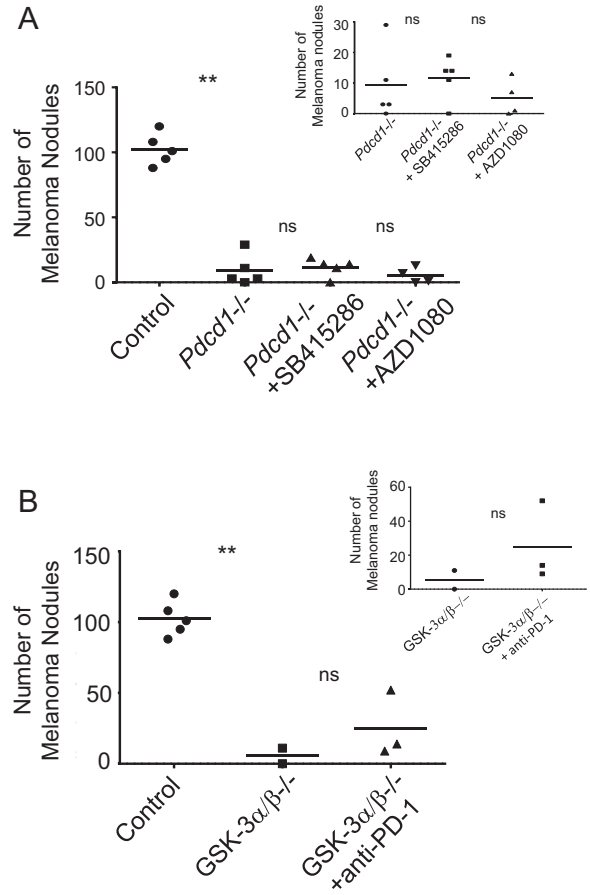

Fig. 3 *Gsk-3−/−* and *Pdcd1−/−* mice show similar resistance to tumor growth. (**a**) *GSK-3α/β −/−* and (**b**) *Pdcd−/−* mice show an identical ability to control the pulmonary metastasis and growth of the B16 melanoma cells. ($n = 3$; number of mice: 5 mice). Upper inset: a more focussed view of tumor nodules in mice. $^{*}P < 0.05$; $^{**}P < 0.001$; *ns* no significant difference relative to controls

combination with other reagents such as anti-CTLA-4 [5, 12, 13]. We have shown that the kinase GSK-3 is a central regulator of PD-1 expression [36] and that the GSK-3 inhibitor SB415286 is as effective as anti-PD-1 in limiting cancer growth [37]. Overall, we show that (i) GSK-3 inhibitor down-regulated PD-1 expression, (ii) SB415286 and anti-PD-1 had similar effects in the control of tumor growth, (iii) GSK-3 inhibitors had no further inhibition of tumor growth in *Pdcd1−/−* mice, and anti-PD-1 failed to reduce B16 pulmonary metastasis in *GSK-3α/β−/−* mice. This observation further supports the notion that GSK-3 inhibition operated to limit tumor growth primarily via the downregulation of anti-PD-1. Since the mice conditionally deleted *GSK-3α/β−/−* in T-cells (i.e., Lck-cre), this result indicated that GSK-3 in the immune system was responsible for the anti-tumor phenotype in mice.

Overall, we have identified a novel pathway that controls the expression of PD-1 in T-cells. The pathway involves GSK-3 inhibition leading to increased T-bet expression followed by its binding and inhibition of *pccd1* transcription and PD-1 expression (Fig. 4). There are potential advantages and disadvantages to the use of GSK-3 inhibitor relative to anti-PD-1 ICB. Anti-PD-1 involves high cost and adverse effects such as fatigue, rash, and possible autoimmune complications. To date, we have seen no evidence of autoimmunity in the GSK-*3α/β−/−* mice over 2 years. The advantage of GSK-3 inhibition includes more accurate dosing, lower cost, and the potential of oral administration. The potential disadvantage of GSK-3 inactivation is a possible effect on the function of other host cells or the tumor itself. However, lithium chloride, an inhibitor of GSK-3, has been used for decades for the treatment of bipolar disease. Further, the suppression of GSK-3 has been reported to directly inhibit the growth of multiple myeloma, neuroblastoma, hepatoma, and prostate tumors [40–44]. It is therefore possible that GSK-3 inhibitors might have an added advantage by directly inhibiting the growth of some tumors in addition to an enhancing effect on the immune system. The use of GSK-3 inhibitors could possibly bypass the impaired proximal TCR signaling events [45, 46]. Further work is needed to uncover the full range of down-stream effects that may be regulated by GSK-3 regulation in anti-tumor immunity.

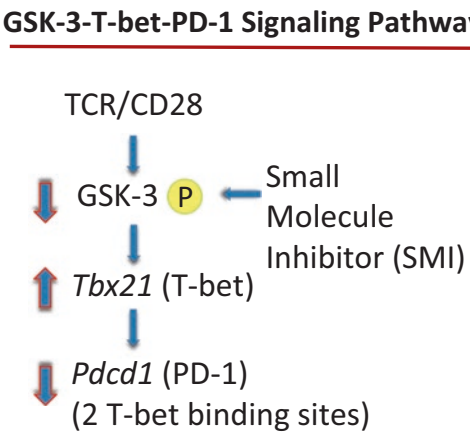

Fig. 4 Model of GSK-3-T-bet-PD-1 Pathway. Model of the novel pathway that controls the expression of PD-1 in T-cells. The pathway involves GSK-3 inhibition leading to increased T-bet expression followed by its binding and inhibition of *Pdcd1* transcription and PD-1 expression

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Index

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