PRECISION MEDICINE IN ONCOLOGY

EDITED BY: Angela Re, Caterina Nardella, Alessandro Quattrone and Andrea Lunardi
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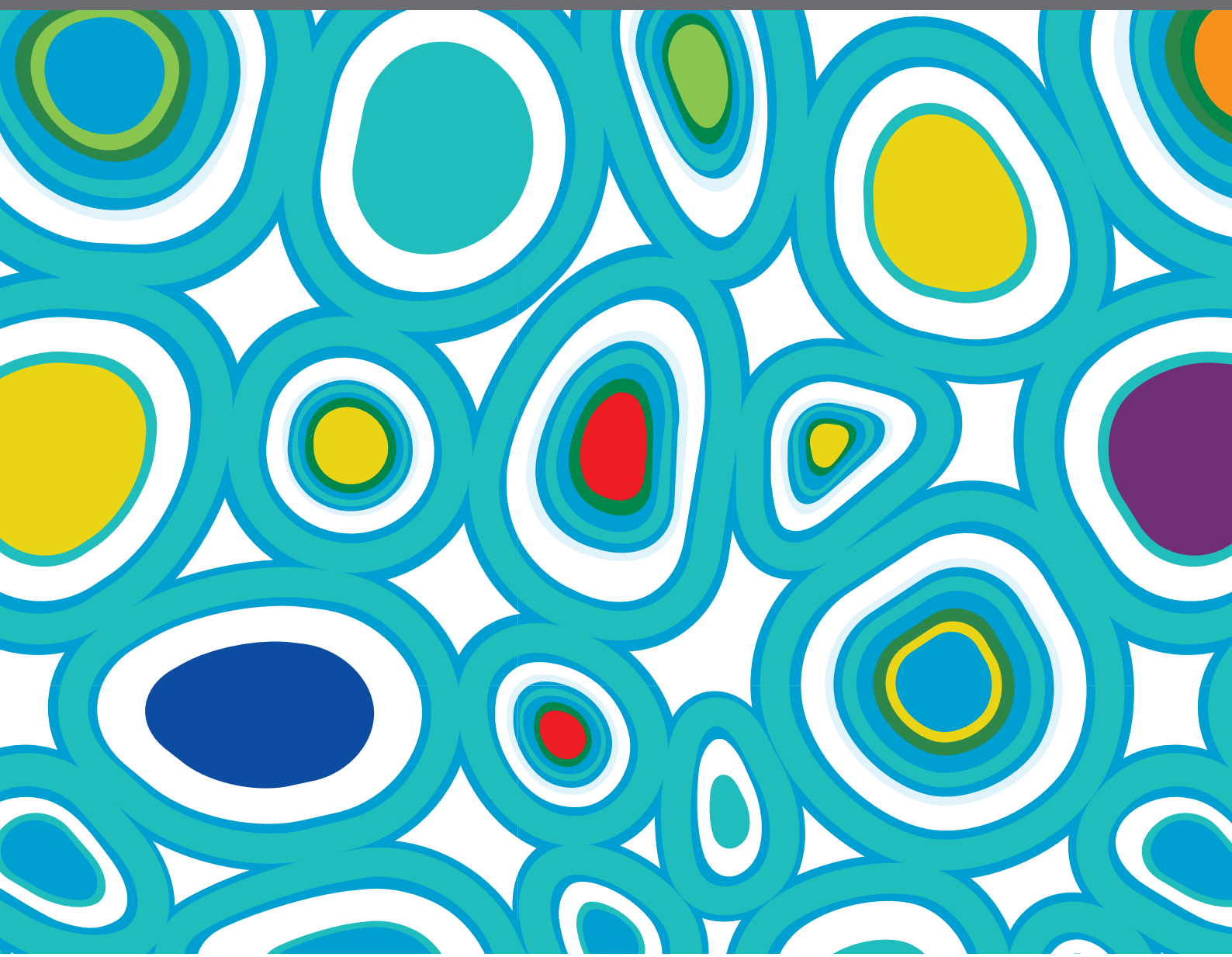

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PRECISION MEDICINE IN ONCOLOGY

Topic Editors:
Angela Re, Fondazione Istituto Italiano di Tecnologia, Italy
Caterina Nardella, University of Trento, Italy
Alessandro Quattrone, University of Trento, Italy
Andrea Lunardi, University of Trento, Italy

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frontiers in Oncology

EDITORIAL
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Editorial: Precision Medicine in Oncology

Angela Re[1]*, *Caterina Nardella*[2], *Alessandro Quattrone*[3] and *Andrea Lunardi*[4]*

[1] Systems and Synthetic Biology Laboratory, Centre for Sustainable Future Technologies, Fondazione Istituto Italiano di Tecnologia, Torino, Italy, [2] Precision Medicine Initiative, Centre for Integrative Biology, Trento, Italy, [3] Translational Genomics Laboratory, Centre for Integrative Biology, Trento, Italy, [4] Armenise-Harvard Cancer Biology & Genetics Laboratory, Centre for Integrative Biology, Trento, Italy

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Editorial on the Research Topic

Precision Medicine in Oncology

Recent advances in technology have unveiled a tremendous heterogeneity in cancer dysfunctional mechanisms. This gain of knowledge has opened a new era in oncology, which relays on the concept that each tumor is different and should be treated in a specific way depending on its distinctive molecular dysfunctions. Fundamental achievements in cancer biology paralleled by unprecedented improvements in disease modeling from all *in silico*, *in vitro* and *in vivo* perspectives, have converged to offer nowadays the compelling opportunity to design therapeutic approaches tailored on individual patients, namely precision medicine.

This Research Topic embodies 13 multidisciplinary manuscripts focused on multifaceted aspects related to "Precision Medicine in Oncology." Overall, each investigator discusses some of the numerous pending issues associated with this ground-breaking field, ranging from basic research findings, novel technologies, and computational approaches to potential innovative translational venues and widely needed new platforms for precision medicine implementation. Specifically, this issue includes: (i) original research reports on novel biological findings and an innovative technology for immunotherapy; (ii) comprehensive reviews on key cancer biomarkers, signaling, and metabolic pathways as well as on theoretical and preclinical models, and analytical integrative methodologies; (iii) insightful perspectives on advanced computational platforms as well as on a novel integrated murine/human clinical infrastructure.

A key aspect for accelerating the development of new effective targeted therapies is represented by a deeper, faster and broader genomic characterization of patient samples. The National Cancer Institute is currently leading numerous multi-disciplinary projects aiming at facilitating the development of precision oncology diagnostics and therapeutic treatments. In a timely review hosted in this Research Topic, Hinkson et al. introduce the Genomic Data Commons (GDC) initiative, which redistributes high quality data and metadata and three Cloud Resources, thus supporting cloud-based access to data, computational scalability and collaboration. Additionally, the review from Davis' group provides an insightful overview on catalogs, software and tools useful for the interpretation of single nucleotide variants and short insertions and deletions in point-of-care high throughput sequencing applications (Tsang et al).

Deep genome and transcriptome sequencing are having two major roles in: (i) facilitating the discovery of new pathways and molecular players involved in cancer onset, progression and drug resistance, thereby offering the opportunity to identify more reliable biomarkers and novel druggable targets; (ii) revolutionizing the clinical approach to human diseases as a result of the unprecedented characterization of the non-coding space of our genome.

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Edited and reviewed by:
Paolo Pinton,
University of Ferrara, Italy

***Correspondence:**
Angela Re
angela.re@iit.it
Andrea Lunardi
andrea.lunardi@unitn.it

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While the non-coding dark matter still remains a challenging target *in vivo*, pharmacological tuning of the coding space has been shown to yield promising results *in vitro* and, to a certain extent, in preclinical and clinical trials. Comunanza and Bussolino describe the insights gained on the vascular-endothelial growth factor (VEGF) over the last 40 years and relevant challenges raised by VEGF-targeted anti-angiogenic therapies (Comunanza and Bussolino). They deeply review the emergence of approaches combining anti-angiogenic regimens with compounds targeting angiogenic mechanisms, oncogenic drivers, and immunotherapy (Comunanza and Bussolino).

In an original research article, Astrologo and colleagues provide evidence that the Bone Morphogenetic Protein 9 (BMP9) might represent a novel therapeutic target in prostate cancer (Astrologo et al). They nicely demonstrate that preventing BMP9 binding to its cell surface receptors, and thus blocking BMP9 signaling, efficiently diminishes prostate cancer cell proliferation and substantially attenuates tumor growth in both an orthotopic model of human prostate cancer and a xenograft derived from an androgen-dependent bone metastatic prostate tumor patient (Astrologo et al).

In respect of non-coding elements, Montironi's group mini review focused on *in vitro* and *in vivo* gain-of-function and loss-of-function experiments showing that long non-coding RNAs play a crucial role in cancer cell invasiveness and metastasis through antagonizing the genome-wide localization and regulatory functions of the SWI/SNF chromatin-modifying complex (Cimadamore et al.) In addition to long non-coding RNAs, another class of regulatory RNAs, namely microRNAs, have been implicated in nearly every signaling pathway. Specifically, microRNA-mediated altered signaling pathway regulation appears to affect a heterogeneous spectrum of cancer behaviors. In this respect, Denti's group provide a comprehensive overview of the tight connection between microRNA misfunction and cancer hallmarks (Detassis et al). They also thoroughly discuss benefits and hurdles of microRNAs as biomarkers to move personalized cancer biogenesis, evolution, diagnosis, and treatment a step forward. Additionally, Gabra and Salmena's review contributes to the debate on the role of microRNAs in personalized cancer therapy focusing on drug resistance and the mechanisms of action that lead to poor overall survival. They also discuss the potential clinical use of miRNA mimic- or antagomir-based approaches in drug resistance overcome (Gabra and Salmena).

Interestingly, two contributors pointed out to the relevance of approaches encompassing metabolism to develop suitable cancer-specific treatments. The extensive crosstalk within and between reactive oxygen species (ROS) detoxification, redox signaling transduction, energy metabolism and central metabolism has been finely reviewed in this Research Topic by Benfeitas et al. The outlined reconstruction of redox metabolism has been connected to the heterogeneity in redox responses displayed by different types of cancer, between individuals affected by the same form of tumor, as well as within different cancer stages. They also highlighted the utility of system-level approaches to capture the role of redox systems in cancer and to design redox-targeting drugs producing synergistic responses for cancer treatment or prevention (Benfeitas et al). On another review, Martín-Martín and colleagues accurately depict the complex interrelationship between metabolism and gene expression regulation in cancer (Martín-Martín et al). The authors report recent advances highlighting how the tight and dynamic coordination between gene expression programs and metabolism dictates cellular adaptations during cancer progression and might lead to new therapeutic opportunities (Martín-Martín et al).

Although counteracting pro-tumorigenic stimuli has always been a major goal in oncology, alternative innovative therapeutic strategies are currently emerging impetuously. Among the most promising ones, we highlight here the synthetic lethality approach and cancer vaccines.

As reviewed by Caffo's group, impairment of DNA damage repair pathways is a common event in cancer, resulting in genomic instability which is crucial for the tumorigenic process (Caffo et al). Exacerbation of such a condition through the administration of DNA damage agents in combination with molecules further affecting DNA repair pathways has been shown to effectively result in cancer cell death (Caffo et al). Importantly, Caffo and colleagues discuss the relevance of applying DNA sequencing approaches for the screening of genomic aberrations affecting DNA repair pathways in prostate cancer with the ultimate goal of stratifying prostate cancer patients for personalized synthetic lethal therapeutic approaches (Caffo et al).

In an original research article, Grandi's group explore the applicability of the Outer Membrane Vesicles (OMVs) platform technology in cancer vaccination (Grandi et al). Technological promising aspects of OMVs, such as the rapidity they can be decorated with foreign epitopes, the high yield production from bacterial fermentation and the easy purification process, inspired the authors to test OMVs amenability for cancer vaccines. Immunization with OMVs engineered with the B cell cancer-specific epitope strongly protected mice from tumor development once injected with a syngeneic cancer cell line expressing the epitope on its surface (Grandi et al). Finally, the synergistic protective activity of multiple epitopes administered with OMVs was found to potentiate the overall efficacy of the OMV cancer vaccine (Grandi et al).

From a clinical perspective, our deeper understanding of oncogenic mechanisms has recently begun to have a crucial impact on clinical decisions at several steps, from cancer prevention and diagnosis to therapeutic intervention. Nowadays, the development of innovative investigational *in silico*, *in vitro,* and *in vivo* platforms fostering the clinical translational potential of basic research findings is of primary relevance.

In this Research Topic, Re reviews significant advancements in our capabilities to tailor synthetic genetic circuits to specific applications in tumor diagnosis, tumor cell- and gene-based therapy, and drug delivery (Re). From a different perspective, Clohessy and Pandolfi present the Mouse Hospital and the Co-Clinical Trial Project focused on the integration of data collected from cancer patients and faithful cancer mouse models enrolled

in concomitant trials (co-clinical trials) with identical treatment protocols. They discuss how co-clinical studies can quickly lead to effective clinical decisions by predicting patients' drug response on genetic and molecular bases as well as by anticipating effective second line treatments for drug resistance-driven cancer relapse (Clohessy et al).

Altogether, the original articles, reviews and perspectives collected in this Research Topic represent an invaluable resource of insights on important achievements attained so far in identifying altered molecular events that lead to the development of cancer and therapy resistance as well as novel therapeutic strategies for the successful delivery of precision medicine approaches in oncology.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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PERSPECTIVE
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A Comprehensive Infrastructure for Big Data in Cancer Research: Accelerating Cancer Research and Precision Medicine

Izumi V. Hinkson[1,2], Tanja M. Davidsen[1], Juli D. Klemm[1], Ishwar Chandramouliswaran[3], Anthony R. Kerlavage[1] and Warren A. Kibbe[1,4]*

[1] Center for Biomedical Informatics and Information Technology, National Cancer Institute, Rockville, MD, United States, [2] Science and Technology Policy Fellowship Program, American Association for the Advancement of Science, Washington, DC, United States, [3] Office of Genomics and Advanced Technologies, National Institute of Allergy and Infectious Diseases, Bethesda, MD, United States, [4] Department of Biostatistics and Bioinformatics, Duke University School of Medicine, Durham, NC, United States

Advancements in next-generation sequencing and other -omics technologies are accelerating the detailed molecular characterization of individual patient tumors, and driving the evolution of precision medicine. Cancer is no longer considered a single disease, but rather, a diverse array of diseases wherein each patient has a unique collection of germline variants and somatic mutations. Molecular profiling of patient-derived samples has led to a data explosion that could help us understand the contributions of environment and germline to risk, therapeutic response, and outcome. To maximize the value of these data, an interdisciplinary approach is paramount. The National Cancer Institute (NCI) has initiated multiple projects to characterize tumor samples using multi-omic approaches. These projects harness the expertise of clinicians, biologists, computer scientists, and software engineers to investigate cancer biology and therapeutic response in multidisciplinary teams. Petabytes of cancer genomic, transcriptomic, epigenomic, proteomic, and imaging data have been generated by these projects. To address the data analysis challenges associated with these large datasets, the NCI has sponsored the development of the Genomic Data Commons (GDC) and three Cloud Resources. The GDC ensures data and metadata quality, ingests and harmonizes genomic data, and securely redistributes the data. During its pilot phase, the Cloud Resources tested multiple cloud-based approaches for enhancing data access, collaboration, computational scalability, resource democratization, and reproducibility. These NCI-led efforts are continuously being refined to better support open data practices and precision oncology, and to serve as building blocks of the NCI Cancer Research Data Commons.

Keywords: genomics, proteomics, imaging, big data, cancer, precision medicine, cloud infrastructure

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Edited by:
Angela Re,
University of Trento, Italy

Reviewed by:
Fahd Al-Mulla,
Genatak, Kuwait
Lawrence Schook,
University of Illinois at Chicago, United States

***Correspondence:**
Anthony R. Kerlavage
anthony.kerlavage@nih.gov

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INTRODUCTION

Precision medicine has evolved out of the seminal work of the Human Genome Project, advancements in DNA sequencing technology, developments in high throughput and large-scale molecular biology technologies, improvements in the speed and scale of computation, and innovations in biomedical informatics. This progress has resulted in the molecular characterization

of individual patient tumors, the identification of actionable genetic alterations, and the development of evidence-based molecular cancer diagnostics and targeted therapies. Although, cancer types have been traditionally classified by organ or cell type, with the aid of genomics, cancer patients are increasingly being treated according to their cancer's unique molecular signature. Cancer is a diverse array of genetically-driven diseases. The identification and validation of actionable genetic alterations including amplifications, rearrangements, and gain-of-function mutations, has spurred the use of genomic data in oncology practices. Targeted gene sequencing panels, for example, offer insight into the genetic drivers of an individual's tumor and inform the diagnosis, prognosis, and targeted treatment of cancer patients. A number of targets for drug development have been outlined previously (Hyman et al., 2017). Imantinib—a BCR-ABL inhibitor for chronic myelogenous leukemia, trastuzumab—a monoclonal antibody-based treatment for HER-2 positive breast cancer, vermurafenib—a mutated BRAF V600E inhibitor for metastatic melanoma, and many others serve as precision oncology success stories. Other candidate genes are currently under pre-clinical and clinical investigation for the development of targeted cancer therapies. Increasing our understanding of how molecular signatures are associated with treatment outcomes in patient populations, and translating these discoveries into the clinic, will improve treatment decisions for the individual.

In support of NCI's Precision Medicine in Oncology Initiative and the Beau Biden Cancer Moonshot, NCI is leading numerous multi-disciplinary efforts to accelerate the development of precision oncology diagnostics and treatments. Here, we describe a subset of ongoing NCI programs that combine biomedical big data, biotechnology, informatics, clinical research, and computer science to create new ways to more precisely study, predict, diagnose, and treat cancers.

NCI PROGRAMS PROVIDE BIG DATA RESOURCES TO SERVE THE CANCER RESEARCH COMMUNITY

The goal of precision oncology is to use each patient's unique collection of germline variants and somatic mutations to inform their diagnosis, prognosis, and therapy; working toward this goal, there has been a push toward large-scale, high throughput studies of patient-derived biospecimens.

Molecular profiling of patient-derived samples, including whole genome sequencing, has led to a data explosion that is contributing to our increased understanding of cancer driver genes, cancer molecular subtyping, cancer risk, therapeutic response, and treatment outcomes. NCI-supported programs such as The Cancer Genome Atlas (TCGA), Therapeutically Applicable Research to Generate Effective Treatments (TARGET), and Clinical Proteomic Tumor Analysis Consortium (CPTAC) have generated large datasets amassing petabytes of data. These, along with other datasets and resources described in this paper are available to researchers both in the US and internationally (**Table 1**).

In December 2005, TCGA was announced as a new collaboration between the NCI and the National Human Genome Research Institute (NIH, 2005). Building upon the pioneering work of the Human Genome Project, the two institutes embarked on a mission to explore the genomic changes that occur in human cancers. The overarching goal of TCGA was to increase our understanding of different cancer types to improve screening and treatments, and to build on this data to create new prevention strategies. TCGA includes the genomic analysis of 33 different tumor types and matched normal tissue from over 11,000 patients and has resulted in thousands of publications (Cancer Genome Atlas Research Network, 2008, 2011, 2012, 2017; Cancer Genome Atlas, 2012). Data types collected include DNA copy number arrays, DNA methylation, exome, and whole genome sequencing, mRNA arrays, microRNA sequencing, and reverse phase protein arrays, totaling ∼2.5 petabytes of data.

TARGET was launched in 2006. TARGET's goal is to characterize the genome and transcriptome of hundreds of pediatric acute lymphoblastic leukemia, acute myeloid leukemia, Wilms tumor, clear cell sarcoma of the kidney, rhabdoid tumor, neuroblastoma, and osteosarcoma samples. Through genomic and transcriptomic analyses, researchers are studying the relationships among alterations at the DNA and RNA levels, cancer growth, cancer progression, and pediatric patient survival (Mullighan et al., 2009; Pugh et al., 2013; Eleveld et al., 2015). The TARGET project has performed whole genome sequencing on most samples collected and the entire dataset is in the petabytes range.

NCI's CPTAC aims to interrogate cancers at the protein level to link genotype to proteotype, with the goal of understanding the basis of cancer phenotypes. CPTAC's objectives are four-fold: (1) characterize the proteomes of tumor and normal tissues; (2) perform proteogenomic analyses of cancer biospecimens; (3) identify potential biomarker candidates through discovery proteomics and develop targeted assays against those candidates; and (4) perform verification tests on those targeted assays. Phase I of CPTAC consisted of technical quality assurance studies (Paulovich et al., 2010). Complementary to TCGA studies, CPTAC Phase II consisted of mass spectrometry-based proteomic analyses of TCGA breast, ovarian, and colorectal samples (Zhang et al., 2014, 2016; Mertins et al., 2016). The recently launched CPTAC Phase III is a proteogenomic analysis of prospectively collected tissues from additional cancer types. Furthermore, to support precision oncology, CPTAC Phase III has established Proteogenomic Translational Research Centers that will study the efficacy of cancer therapies on individual tumor samples to generate predictive models. CPTAC data currently totals ∼16 TB of data, and upon completion of CPTAC III, this number is expected to increase four-fold to ∼66 TB of data.

With the announcement of the Beau Biden Cancer Moonshot, the Applied Proteogenomics Organizational Learning and Outcomes (APOLLO) Network has emerged as a tri-agency collaboration to enable oncologists to use their patients' unique proteogenomic profiles to inform precision oncology treatments (Moonshot, 2016; OCCPR, 2016). Together with the Department of Veterans Affairs (VA) and the Department of Defense (DoD),

TABLE 1 | Selected NCI-supported projects.

Project name	Lead institution(s)	Project URL
The Cancer Genome Atlas (TCGA)	National Cancer Institute National Human Genome Research Institute	cancergenome.nih.gov
Therapeutically Applicable Research to Generate Effective Treatments (TARGET)	NCI Office of Cancer Genomics	ocg.cancer.gov/programs/target
Clinical Proteomic Tumor Analysis Consortium (CPTAC)	NCI Office of Cancer Clinical Proteomics Research	proteomics.cancer.gov/programs/cptac
Applied Proteogenomics Organizational Learning and Outcomes (APOLLO) Network	Department of Defense Department of Veterans Affairs National Cancer Institute	proteomics.cancer.gov/programs/apollo-network
The Cancer Imaging Archive (TCIA)	University of Arkansas for Medical Sciences NCI Division of Cancer Treatment and Diagnosis	www.cancerimagingarchive.net
Genomic Data Commons (GDC)	NCI Center for Cancer Genomics	gdc.cancer.gov
Database of Genotypes and Phenotypes (dbGaP)	National Center for Biotechnology Information	www.ncbi.nlm.nih.gov/gap
NCI Cloud Resources	National Cancer Institute	cbiit.cancer.gov/cloudresources
Broad FireCloud	Broad Institute	firecloud.org
Institute for Systems Biology Cancer Genomics Cloud (ISB-CGC)	Institute for Systems Biology	isb-cgc.org
Seven Bridges Cancer Genomics Cloud (SB-CGC)	Seven Bridges	www.cancergenomicscloud.org
NCI Cancer Research Data Commons	National Cancer Institute	cbiit.cancer.gov/cancerdatacommons

NCI-supported projects annotated with lead institutions and URLs.

NCI aims to perform proteogenomic analyses of a cohort of 8,000 cancer patients within the VA and DoD healthcare systems. These analyses will provide insight into the mutations and pathways that drive cancer progression and support the development of targeted and combination therapies. In the next 5 years, APOLLO is expected to amass petabytes of genomic, proteomic, imaging, and clinical data.

As the -omics sciences increase the volume of data collection, the need for big data solutions intensifies. To address this need, biomedical research has been moving toward data curation and data sharing models established by other big data fields such as astrophysics. Through major technological advancements, the Hubble Deep Field image marked a turning point in astrophysics where researchers led a concerted effort in data quality assessment, annotation, and curation. This work led to the development open source data resources, and user interfaces that obviated the resource intensive download of large datasets (Andersen, 2012). Biomedical informatics has reached a similar a turning point where key innovations in data storage and distribution such as compression algorithms, indexing systems, and cloud platforms must be leveraged.

NCI GENOMIC DATA COMMONS AND CLOUD RESOURCES

In addition to the data curation and storage needs of modern biomedical research, other challenges include the development of robust analytical tools, as well as infrastructure and funding models to support these efforts. As data generation expands, local storage, and computational solutions become less feasible. Thus, NCI has set out to build the NCI Cancer Research Data Commons (NCRDC), a cloud-based infrastructure in support of data sharing, tool development, and compute capacity to democratize big data analysis and to increase collaboration among researchers. NCI has sponsored two recent initiatives that serve as the foundation for the Cancer Research Data Commons—the Genomics Data Commons (GDC), and three Cloud Resources.

The GDC, built and managed by the University of Chicago Center for Data Intensive Science, in collaboration with Ontario Institute for Cancer Research, all under an NCI contract with Leidos Biomedical Research, is a unified genomic data repository that hosts authoritative NCI reference datasets such as TCGA and TARGET (Grossman et al., 2016; NIH, 2016). The primary goals of the GDC are to ensure data and metadata quality, ingest and harmonize genomic data, support data dissemination practices in alignment with Findable Accessible Interoperable Reusable (FAIR) principles (Mons et al., 2017), and securely redistribute data to researchers. In addition, the GDC takes part in collaborative efforts such as the Global Alliance for Genomics and Health (Knoppers, 2014). Through the GDC, researchers can download harmonized genomic data for analysis on their local servers. To bolster data sharing practices and streamline genomic data analysis, much of the genomic data stored at the GDC have been made available through the NCI Cloud Resources.

The NCI Cloud Resources were initially launched in 2016 as the Cancer Genomics Cloud (CGC) Pilots. The purpose of the CGC Pilots was to explore multiple cloud-based approaches for enhancing secure data access, collaboration, computational scalability, resource democratization, and reproducibility. Through this program, the Broad Institute, the Institute for Systems Biology, and Seven Bridges have each developed what are now known as Cloud Resources. Each platform is deployed in a commercial cloud, and has applied a distinct approach to providing access to TCGA and TARGET genomic data in a cloud environment, and integrating proteomic data from CPTAC as well as radiology images and associated metadata from The Cancer Imaging Archive (TCIA). In addition to providing access to these datasets through rich Application Programming Interfaces (APIs) and graphical user interfaces, the Cloud Resources each provide a platform to enable the deployment of analysis, visualization, and other computational tools in the cloud, bypassing the need to bring data to a local infrastructure. The Cloud Resources support tool deployment through the use of Docker containers, which allow users to package their tools along with all associated dependencies. These "containerized" tools can be connected and executed as workflows in these cloud environments. End user documentation provides users with guidance on how to query data, install tools, as well as create and run workflows in each environment. All three platforms conform to strict federal information system security requirements and manage access to controlled data through Database of Genotype and Phenotype (dbGaP) authorization. In addition to their fundamental charter of providing secure cancer genomic data access co-localized with analysis pipelines and visualization tools, the Cloud Resources each offer unique capabilities suitable for a range of research needs.

Broad Firecloud

The Broad Institute's FireCloud, was built as the next generation of Broad Institute's Firehose data analysis infrastructure developed for the TCGA program (Ulrich, 2016). FireCloud harnesses the elastic compute capacity of Google Cloud Platform for large-scale genomic analyses akin to those available through Firehose. Key advantages offered by FireCloud include running Broad's best practice tools and pipelines such as ContEst, MuTect, and Oncotator. FireCloud users can also access curated open and controlled-access TCGA workspaces, upload their own data, and share workspaces with collaborators. FireCloud also allows users to leverage the rich query interface of the GDC to create cohorts of interest and download data "just-in-time" to a FireCloud-based workspace for follow on analyses. Similar approaches are under development to support the analysis of CPTAC data and TCIA images. Researchers at the Broad Institute, in collaboration with IBM Watson, are using FireCloud to tackle one of precision oncology's toughest questions—which genomic signatures are linked to drug-resistant cancers (Park, 2016)? While targeted therapies are currently being applied in the clinic, oncologists have been unable to predict when a patient will no longer respond to a given line of therapy. The data analysis infrastructure provided by FireCloud directly supports researchers investigating problems such as this one to increase the efficacy of precision medicine for cancer patients.

ISB-CGC

The Institute for Systems Biology Cancer Genomics Cloud (ISB-CGC) runs on the Google Cloud Platform and offers an interactive web-based application and hosts Application Programming Interfaces (APIs) such as the Global Alliance for Global Health API. ISB-CGC takes advantage of Google Cloud Platform's built-in resources such as BigQuery, Compute Engine, App Engine, Cloud Datalab, and Google Genomics. Researchers can use BigQuery to explore clinical, biospecimen, level-3 open access TCGA, and CPTAC II data. ISB-CGC hosts numerous genomics tools and has recently added the Trans Proteomic Pipeline analysis suite. Researchers can now access complementary genomic and proteomic data, run multi-omic analyses, and perform BigQuery searches to investigate genetic alterations, copy number, transcript expression, protein expression, and molecular pathways that are involved in cancer biology. ISB-CGC has also made radiology and tissue images from TCIA and the GDC available through Google Cloud Storage. Additional datasets available at ISB-CGC include the Catalog of Somatic Mutations in Cancer[1] and the Cancer Cell Line Encyclopedia (CCLE)[2]. A recent publication in Nature Scientific Reports showcased a project which used the ISB-CGC to perform fast, cheap, and robust RNA-sequencing analyses of 12,307 samples from CCLE and TCGA (Tatlow and Piccolo, 2016). Authors, P. J. Tatlow and Dr. Stephen Piccolo, used preemptible virtual machines to analyze over 64 terabytes of TCGA data for only $0.09 per sample. The scalable, cost effective compute capabilities of ISB-CGC have enabled researchers to perform robust analyses of big data that will ultimately lead to the enhanced understanding of individual cancers.

SB-CGC

Currently, over 1,600 researches from over 40 countries are using the Seven Bridges Cancer Genomics Cloud (SB-CGC) to analyze hosted genomic data, and/or their own data[3]. Dr. Julia Salzman's lab at Stanford University has deployed Mismatched Alignment CHimEra Tracking Engine (MACHETE) (Hsieh et al., 2017), a statistical algorithm for the detection of gene fusions, on the SB-CGC (Salzman, 2017). Using RNA-seq data from hundreds of TCGA samples, MACHETE was used to perform statistical modeling of fusion artifacts to precisely detect novel gene fusions including rare potential drivers of cancer. This research, fueled by cloud computing, is enabling precision oncology through the discovery of novel, potentially druggable gene fusions. In addition to the TCGA and TARGET data, SB-CGC hosts TCGA radiology images, CCLE data, as well as Simons Genome Diversity Project data[4]. Leveraging its Cancer Genomic Cloud work, Seven Bridges has partnered with the Blood Profiling Atlas in Cancer Consortium[5], to develop the Blood Profiling

[1] cancer.sanger.ac.uk/cosmic
[2] portals.broadinstitute.org/ccle
[3] http://www.cancergenomicscloud.org/usage
[4] docs.cancergenomicscloud.org/docs
[5] http://www.bloodpac.org

Atlas Analysis Cloud and provide the research community with analysis algorithms for liquid biopsy.

CGC Pilot Beta-Testing and Evaluation

The initial versions of The NCI Cloud Resources, the CGC Pilots, were created to maximize the value of cancer -omics data through harnessing multi-disciplinary expertise. Synergizing technologies from the fields of medicine, molecular biology, informatics, and cloud computing, the CGC Pilots have begun to transform how cancer data analysis is conducted. Researchers, both in the US and internationally, have been able to advantage of these cloud-based resources. To ensure the success of this project and to identify areas for improvement, the CGC Pilot and NCI teams established mechanisms to support early adopters' use of these platforms and to collect their feedback.

The CGC Pilots teams provided technical support to new users who sought to implement new tools, access data, or create collaborative workspaces. Through the three CGC Pilots, NCI provided cloud compute and storage "credits" to offset the costs of evaluation of these platforms by cancer researchers. These funds directly impacted the work of researchers such as post-doctoral scholar Dr. Brittany Lasseigne, at the HudsonAlpha Institute for Biotechnology in Huntsville, AL. Dr. Lasseigne used the SB-CGC, to study dosage effects, context dependency, and tissue specificity of tumor suppressors across human cancers in TCGA. These Cloud credits supported the use of the large-scale genomic datasets co-located with computational resources and analysis tools, and increased research efficiency for many early stage researchers like Dr. Lasseigne (Lasseigne, Personal Communication).

To evaluate the CGC Pilots and support on-going NCI-funded cancer research, NCI funded administrative supplements to the active grants of investigators performing genomics-based research. The Funding Opportunity Announcement, Supplements to Support Evaluation of the NCI Cancer Genomics Cloud Pilots (PA-15-305), funded projects to use one or more of the NCI CGC Pilots for ongoing research activities. Funds were awarded to investigators whose projects aimed to install and test the performance of new analysis tools on a CGC Pilot, upload locally-generated genomic data and

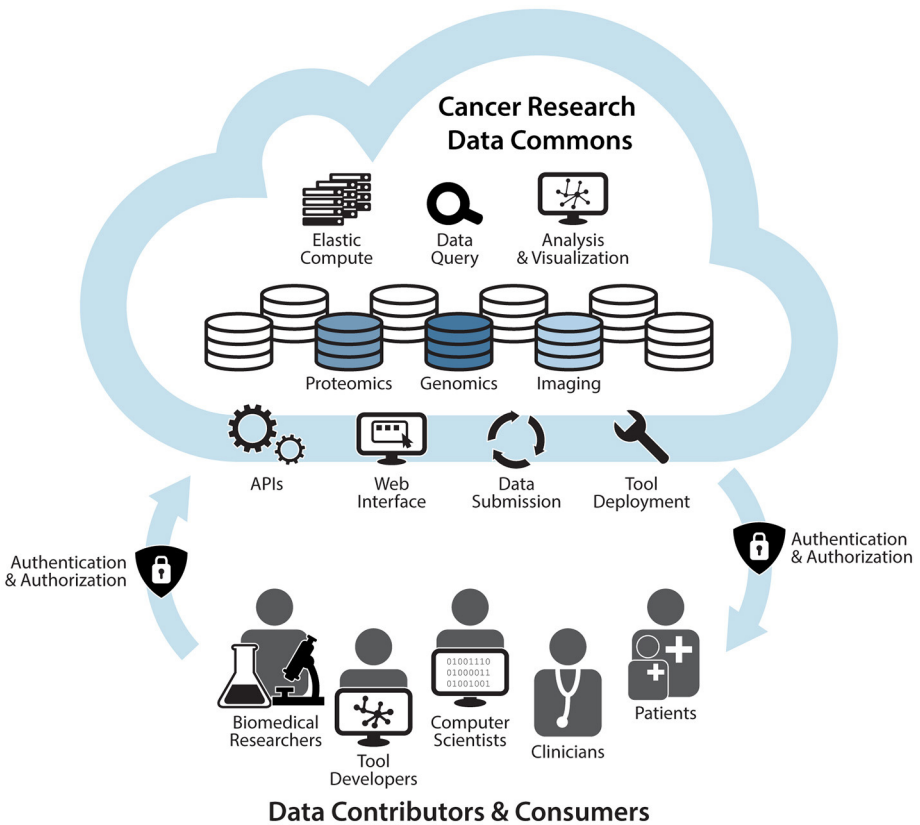

FIGURE 1 | The NCI Cancer Research Data Commons: An Expandable Infrastructure. The NCI Cancer Research Data Commons will be a cloud-based network in which each node is focused on a specific data type. Nodes will include the Genomic Data Commons, Proteomic Data Commons, and Imaging Data Commons. Future plans include the addition of nodes that support other research modalities such as clinical data, epidemiological data, and cancer models. Through a secure authentication and authorization process, biomedical researchers, tool developers, computer scientists, informaticians, clinicians, and patients will be able to bring their own data and tools to nodes, as well as access harmonized data and hosted tools via APIs and a web interface. Users will also be able to harness elastic compute capabilities for computational analyses, visualization of results, and data queries in the cloud (NCI, 2017).

perform analyses on a CGC Pilot, and/or perform analyses of hosted TCGA data. The researchers reported having a generally positive experience working on the CGC Pilots; however, as expected, some encountered technical hurdles. When those technical issues arose, the vast majority of the groups were able to resolve their problems by working directly with the CGC Pilot support teams. Each research group provided extensive feedback to the CGC Pilots and NCI teams on what elements of the CGC Pilots could be improved. The majority of the administrative supplement awardees reported that they plan to continue to use the CGC Pilots to accelerate their research and that the CGC Pilots have the potential to be a vital resource for the cancer research community. The activities and outcomes of projects funded through these supplements have helped inform NCI's decision to continue supporting this project beyond the pilot phase and to develop a more comprehensive computational infrastructure for -omics and other big data types.

Future Vision: NCI Cancer Research Data Commons

Cancer research in the era of big data presents major challenges: computing on large datasets, combining expertise from various disciplines, and developing the infrastructure needed to enhance research efficiency. Recognizing the importance and urgency of these needs, the Beau Biden Cancer Moonshot Blue Ribbon Panel has recommended that the cancer research community aim to, "collect, share, and interconnect a broad array of large datasets so that researchers, clinicians, and patients will be able to both contribute and analyze data, facilitating discovery that will ultimately improve patient care and outcomes (BRP, 2016)." In line with this recommendation, the NCI is taking steps toward establishing the NCRDC, with the GDC and the Cloud Resources serving as the foundation for this vision.

The GDC and Cloud Resources currently support basic and translational research, primarily using genomic and clinical data. These activities serve as the building blocks of the cloud-based NCRDC (**Figure 1**). The NCRDC will consist of multiple "nodes," or digital knowledge bases with functionalities like those of the GDC and Cloud Resources. NCRDC nodes will each be centered on different research and clinical data types such as genomics, proteomics, imaging, cancer models, and epidemiology. Each node will house annotated datasets, raw data files, metadata, analysis, and visualization tools, as well as individual and collaborative workspaces. NCRDC users will be able to access authoritative datasets generated by NCI funded programs such as TCGA, TARGET, CPTAC, APOLLO, and TCIA. Each node will also employ a standardized process for data submission and quality control that will allow for the harmonization of new data, including user-generated data. Containerized tool deployment will also be supported by each Data Commons node. Each node will provide consistent, well-defined identifiers and semantics for access to data housed in that node and provide broadly-available computational support critical to the demands of modern cancer research and precision oncology. The Data Commons will thus support cancer research across multiple domains and platforms, allow for these data to be queried and analyzed in an integrated, secure, cross-domain manner, and provide the mechanisms for new data sources to be incorporated as they are generated. Through fostering community-driven, open-development informatics initiatives, the Cancer Research Data Commons will create, maintain, and extend informatics infrastructure and standards to improve connectivity among disparate information systems. Combining innovation, cloud computing, big data, and FAIR principles, this robust infrastructure will provide significant support for NCI's Precision Medicine in Oncology Initiative and the Beau Biden Cancer Moonshot by accelerating the discovery of novel therapeutic targets and disease biomarkers for individual cancer patients.

The era of big data in biomedical research and precision oncology calls for creative strategies borrowed from multiple scientific and technological disciplines. The GDC and Cloud Resources are important steps in supporting the next generation of data-driven cancer research. Looking ahead, the NCRDC represents an interdisciplinary solution to the challenges of big data in cancer research. NCI will continue to lead open science efforts toward the goals of improving prevention strategies, developing targeted diagnostics and therapeutics, and reducing the burden of cancer on patients, their families, and society.

AUTHOR CONTRIBUTIONS

IH: Manuscript writing, figure design. TD, JK, AK, and WK: Manuscript and figure revision, approval of final manuscript. IH, TD, JK, IC, AK, and WK: Management and project support for Cancer Genomics Cloud Pilots.

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REVIEW
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Resources for Interpreting Variants in Precision Genomic Oncology Applications

*Hsinyi Tsang[1,2], KanakaDurga Addepalli[1,2] and Sean R. Davis[3]**

[1] Center for Biomedical Informatics and Information Technology, National Cancer Institute, National Institutes of Health, Gaithersburg, MD, United States, [2] Attain, LLC, McLean, VA, United States, [3] Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, United States

Precision genomic oncology—applying high throughput sequencing (HTS) at the point-of-care to inform clinical decisions—is a developing precision medicine paradigm that is seeing increasing adoption. Simultaneously, new developments in targeted agents and immunotherapy, when informed by rich genomic characterization, offer potential benefit to a growing subset of patients. Multiple previous studies have commented on methods for identifying both germline and somatic variants. However, interpreting individual variants remains a significant challenge, relying in large part on the integration of observed variants with biological knowledge. A number of data and software resources have been developed to assist in interpreting observed variants, determining their potential clinical actionability, and augmenting them with ancillary information that can inform clinical decisions and even generate new hypotheses for exploration in the laboratory. Here, we review available variant catalogs, variant and functional annotation software and tools, and databases of clinically actionable variants that can be used in an *ad hoc* approach with research samples or incorporated into a data platform for interpreting and formally reporting clinical results.

Keywords: precision oncology, high throughput sequencing, genomic variation, cancer variants, precision medicine, databases, genetic

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Angela Re,
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Istituto Europeo di Oncologia, Italy

****Correspondence:***
Sean R. Davis
seandavi@gmail.com

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1. INTRODUCTION

Genomic technologies and approaches have transformed cancer research and have led to the production of large-scale cancer genomics compendia (1, 2). The resulting molecular characterization and categorization of individual samples from such compendia has driven development of molecular subtypes cancers as well as enhanced understanding of the molecular etiologies of carcinogenesis (3–5). The development of novel and effective targeted therapies has proceeded in parallel with and been accelerated by deeper, faster, and broader genomic characterization (6), enabling early application of molecular characterization at the point of care to inform clinical decision-making (7–10) and to address resistance to primary therapy (11). Genomic characterization also has applications in immune approaches to cancer. For example, chimeric antigen receptor T-cell (CARt) therapy has shown great success in diseases with well-characterized antigens that are relatively tumor-specific (12) as identified by genomic profiling. Variously referred to as precision oncology (13), genomics-driven oncology (14), genomic oncology, and even simply as precision medicine, the paradigm

of applying high-throughput genomic approaches to patient samples is rapidly changing the landscape of oncology care and clinical oncology research.

Conventional approaches to clinical trials design may be inadequate due to molecular heterogeneity of tumors derived from a single primary tissue (15), leading to the adoption of basket, umbrella, and hybrid trials designs. A number of studies are ongoing to determine feasibility and potential impact of precision genomic oncology at the point-of-care (16–18). In addition to studies focused on identifying targetable mutations, immune-based therapeutic approaches are also being informed by HTS applied to patient samples (19–21).

One of the most recent developments in the field of precision oncology is the approval of Pembrolizumab (Keytruda), an anti-PD-1 antibody that functions as a checkpoint inhibitor, by the US Food and Drug Administration for treatment of solid tumors that show genetic evidence of mismatch repair and, therefore, carry very high mutational burdens (22). Pembrolizumab was previously approved for use in melanoma, but the most recent approval is the first that is targeting allows a drug to be used in a non-tissue-specific context in patients showing a specific genomic marker in any solid tumor (23).

As with any clinical testing modality, whether in a research setting or at the point-of-care, a clear understanding of the goals of applying the test is necessary when first designing the test and its validation. However, the flexibility and number of potential data items that arise from even a limited application of HTS has lead the US Food and Drug Administration (FDA) to begin to define its regulatory role (24) and, critically, how existing knowledge bases can be applied in real time to address findings from clinical HTS testing (25).

This review aims to provide an organized set of biological knowledge bases with relevance to the interpretation of small variants, defined as single nucleotide variants or short (on the order of 20 base pairs or fewer) insertions and deletions. The catalogs of observed variants section list large-scale catalogs of variants, useful for filtering known common polymorphisms and identifying previously identified cancer variants. When a variant observed in a clinical sample has not been seen but appears to affect the protein coding sequence, the functional annotation resources section presents a sampling of some of the most common software and databases for predicting the impact on protein function. Finally, we catalog several data products and knowledgebases have been developed to provide decision support (with strong disclaimers and caveats) directly linking observed variants to clinical intervention in point-of-care HTS applications. Integrating the various data sources described in this review with variants observed in individual patients can be accomplished with combinations of software tools for the manipulation of variant datasets.

1.1. Catalogs of Observed Germline and Somatic Variants

Databases of observed variation in normal populations, diseased individuals, and cancer compendia form the map onto which observed variants in patients are projected. Because of the vast quantities of genomic data and, specifically, DNA variants, there is a tension between providing rich, highly curated information about individual variants and producing the largest possible catalog of variants with manageable levels of curation. This section reviews some of the available catalogs (**Table 1**) of genomic variation observed in the germline as well as those that appear in tumors as somatic mutations. Note that many of the databases mentioned below overlap in data sources (some nearly completely), but they may differ in the amount and depth of curation, additional metadata added to each variant, speed of updates, and methods or formats for access.

1.2. Germline

Comprehensive catalogs of germline variants inform decisions about the frequency of variants as seen in the general population as well as to identify variants that are annotated as cancer associated. In the context of tumor sequencing, common variants are unlikely to be genomic drivers of carcinogenesis and are often filtered from a report of potential somatic variants. This filtering process is particularly important when tumor sequencing is not accompanied by matched normal sequencing. Additional germline databases that catalog disease-associated variants can be useful to begin to address familial risk and potentially pharmacogenomic loci (38, 39).

Perhaps the oldest of the variant catalogs, dbSNP contains 325,658,303 individual variant records (build 150, accessed

TABLE 1 | Catalogs of germline and somatic variants.

Resource	Variant Type	URL	Reference
dbSNP[a]	Germline and somatic	https://www.ncbi.nlm.nih.gov/projects/SNP/	(26)
COSMIC[a]	Somatic	http://cancer.sanger.ac.uk/cosmic	(27)
ClinVar[a]	Germline predisposition and somatic	https://www.ncbi.nlm.nih.gov/clinvar/intro/	(28)
gnomAD[b]	Germline	http://gnomad.broadinstitute.org/	(29)
69 genomes from CGI[c]	Germline	http://www.completegenomics.com/public-data/69-genomes/	(30)
Personalized Genome Project	Germline	http://www.personalgenomes.org/	(31)
NCI Genomic Data Commons	Germline and somatic	https://portal.gdc.cancer.gov/	(32)
cBioPortal	Somatic	http://www.cbioportal.org	(33, 34)
Intogen (Partial TCGA dataset)	Somatic	https://www.intogen.org/search	(35, 36)
Pediatric Cancer Genome Project	Somatic	http://explorepcgp.org	(37)

The most commonly used catalogs include dbSNP, COSMIC, ClinVar, and gnomAD.

[a]*Primary resources useful for all studies.*

[b]*Particularly useful for exome sequencing projects.*

[c]*Useful if the Complete Genomics platform was used.*

May 30, 2017) and is available in multiple formats, searchable, and linked to records in literature and other data resources and databases. While the vast majority of variants in dbSNP have been observed in individuals without cancer, somatic variants are included and annotated in the database. Because dbSNP is driven by community submission of variants, levels of evidence vary among individual variants. The genome Aggregation Database, or gnomAD (29, 40), contains information from 123,136 exomes and 15,496 whole-genomes from unrelated individuals sequenced as part of various disease-specific and population genetic studies (accessed May 30, 2017). These data were collected by numerous collaborations, underwent standard processing, and unified quality control and results area accessible as a searchable online database and as a downloadable VCF-format text file. ClinVar (28), maintained by the NIH National Center for Biotechnology Information (NCBI), is a freely available archive for interpretations of clinical significance of variants for reported conditions. Entries in ClinVar are taken directly from submitters and represent the relationship between variants and clinical significance. When multiple submissions concerning a single variant are available, ClinVar supplies high-level summaries of agreement or disagreement across submitters. Importantly, though, clinical significance in ClinVar is reported as supplied by the submitter. The Personalized Genome Project (31) provides a limited number of fully open-access genome sequencing results provided by volunteers with trait surveys and even some microbiome surveys of participants. A catalog of germline variants derived from 69 genomes sequenced using the Complete Genomics sequencing platform (30) may be useful for groups who have data generated from the same platform, particularly for identifying sequencing-platform-specific false positive results.

1.3. Somatic

Whereas databases of germline variants are useful to filter out variants unlikely to be directly involved in carcinogenesis, databases of somatic variants are useful to identify variants and their frequencies as observed in tumors. In some cases, identified variants may be associated with specific tumor types, offering mechanistic clues, particularly in the rare cancer setting where biological understanding may be limited.

Several catalogs of somatic variants have, at their core, variants derived from The Cancer Genome Atlas (TCGA). These databases vary in the pipelines used to define the variants, the level of annotation associated with individual variants, the proportion of TCGA included, and methods for accessing or querying. Recently, National Cancer Institute (NCI) has established the Genomic Data Commons (GDC) to harmonize clinical information and genomic results across enterprise cancer datasets (32), particularly those funded by NCI, such as TCGA. In addition to the adult tumors profiled as part of the TCGA, the NCI GDC also contains data from several pediatric tumors profiled as part of the Therapeutically Applicable Research To Generate Effective Treatments (TARGET) project (41). Cancer cell line data from the Cancer Cell Line Encyclopedia (CCLE) are also included (42) in the GDC data collection. The GDC is a modern data platform that provides multiple access methods, including a programmatic application programming interface (API), data file download, and web browser-based text and graphical queries and visualization. The International Cancer Genome Consortium (ICGC) is a large, international collaboration with a collection of 76 studies (including TCGA studies) encompassing 21 tissue primary sites. Like the NCI GDC, the ICGC data portal provides modern data platform approaches to data access, visualization, and query (43). The Catalog of Somatic Mutations in Cancer (COSMIC) database is perhaps the largest and best-known cancer variant database. It presents a unified dataset consisting of curated cancer variants for specific genes as well as genomic screens from projects, such as TCGA. Several other cancer variant data resources are listed in **Table 1**.

2. FUNCTIONAL ANNOTATION RESOURCES

When faced with variants with little or no literature or database support, differentiating those that variants that are likely to be deleterious, perhaps contributing to carcinogenesis, versus those that likely are tolerated by the cell is a critical task, particularly in the setting of clinical precision genomic oncology. Note that determing that a variant is deleterious is not likely to result in a change in diagnosis, prognosis, or therapy. However, prioritizing variants for further study, research interest, and for discussion in forums such as a molecular tumor board is a valuable and necessary aspect of applying genomic technologies in the clinical arena.

A number of algorithms and methods have been developed to predict the effect of observed variants on protein structure and function as well as the potential for clinical impact. These prediction methods utilize features of the variant and its context, such as sequence identity, sequence conservation, evolutionary relationship, protein primary and secondary structure, entropy-based protein stability, and approaches such as clustering based on sequence alignments and machine learning. Some of them are specific to the type of variant or mutation, some to a disease type, and some more general. Therefore, applying these functional annotational tools and interpreting the results in a clinical or research setting may require significant human curation before being recognized as clinically actionable. Here, we present a review of a representative set of approaches for predicting pathogenicity of different variants. For a comprehensive list of prediction tools and their details, see **Table 2**. For more detailed scientific and technical explanations of these methods, we refer the reader to a comprehensive review (44).

2.1. SIFT

Sorting Intolerant From Tolerant, or SIFT, that predicts functional impacts of amino acid substitutions (48) is one of the earliest variant effect prediction tools and represents the class of prediction algorithms that utilizes protein conservation. It has since been updated and an online version of the tool is available (67). SIFT uses sequence homology, as measured by protein-level conservation, to classify variants based as tolerated or deleterious based on the associated protein coding changes. SIFT has served as a benchmark against which other methods are compared because

TABLE 2 | Tools, software, and databases for functional prediction and annotation of variant impact.

Resource	URL	Reference	Notes
Integrated predictive methods and aggregated databases			
dbNSFP[a,b,c,d]	https://sites.google.com/site/jpopgen/dbNSFP	(45)	Aggregated database of variant information
myvariant.info[a]	http://myvariant.info/	(46)	Aggregated database of variant information
Functional effect prediction software and algorithms			
PolyPhen-2[b]	http://genetics.bwh.harvard.edu/pph2	(47)	Bayesian classification
SIFT[b]	http://sift.jcvi.org	(48)	Alignment scores
MutationAssessor	http://mutationassessor.org	(27)	Conservation, naive Bayes classifier
MutationTaster	http://www.mutationtaster.org	(49)	
PROVEAN	http://provean.jcvi.org/index.php	(50)	
CADD[b,c]	http://cadd.gs.washington.edu	(51)	
GERP++[c]	http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html	(52)	
PhyloP and PhastCons	http://compgen.cshl.edu/phast/index.php	(53, 54)	
nsSNPAnalyzer	http://snpanalyzer.uthsc.edu/	(55)	Random Forest
SNPs&GO	http://snps-and-go.biocomp.unibo.it/snps-and-go/	(56)	SVM
SNAP2	https://rostlab.org/services/snap2web/	(57)	Neural Networks
SNPs3D	http://www.snps3d.org/	(58)	Structure and sequence analysis
MutPred2	http://mutpred.mutdb.org/	(59)	Random Forest
AUTO-MUTE	http://binf2.gmu.edu/automute/	(60)	Topology and statistical contact potential
Panther	http://www.pantherdb.org/tools/csnpScoreForm.jsp	(61)	Hidden Markov Model
stSNP	http://ilyinlab.org/StSNP/	(62)	Comparative modeling of protein structure
Condel[b]	http://bg.upf.edu/fannsdb/	(63)	A weighted average of multiple methods
CoVEC	https://sourceforge.net/projects/covec/files		
CAROL[b]	http://www.sanger.ac.uk/science/tools/carol	(64)	Combines PolyPhen-2 and SIFT
Cancer-specific prediction tools			
CHASM	http://wiki.chasmsoftware.org/index.php/Main_Page	(65)	Random Forest
CanDrA	http://bioinformatics.mdanderson.org/main/CanDrA#CanDrA	(66)	96 structural, evolutionary and gene features

[a]*Aggregated databases combine outputs of other databases and algorithms are, therefore, efficient resources to use in annotation pipelines. Adding these resources to observed variants is supported software in* ***Table 4*** *including Ensembl VEP software (noted[b] in this table), Annovar (noted[c]), and snpEff (noted[a]).*

of its relative simplicity. SIFT considers the type of amino acid change induced by a genomic variant and the position at which the change/mutation occurs. SIFT relies on the presence of sequences from which conservation can be determined; variants for which such databases are limited will potentially lack robust predictions.

2.2. PolyPhen-2

Polymorphism Phenotyping v2, or PolyPhen2, predicts the effecting of coding non-synonymous SNPs on protein structure and function and annotates them (47). This algorithm uses a naive Bayes approach to combine information across a panel of 3D structural, sequence-based, and conservation-based features. Trained on two datasets, HumDiv and HumVar, and associated non-deleterious controls, the PolyPhen2 algorithm represents a class of multivariate prediction algorithms that employ machine learning and multiple features of variant impact.

2.3. Mutation Assessor

Mutation Assessor is an algorithm and tool that, such as SIFT, uses a conservation-based approach. However, Mutation Assessor also incorporates evolutionary information in an attempt to account for shifts in function between subfamilies of proteins (27), potentially extending the functional annotation of variants to "switch of function" as well as loss or gain of function. By quantifying the impact to conserved residues both globally and within subfamilies (residues that distinguish subfamilies from each other are thought to be less tolerant to change), Mutation Assessor defines a functional impact score to predict which variants are likely to be deleterious.

2.4. CONDEL

The CONsensus DELeteriousness, or CONDEL score, is an integrated prediction method for missense mutations that is relatively easy to extend with additional prediction resources (63). Originally implemented as a weighted average of the normalized scores from the output of two computational tools, Mutation Assessor and FATHMM, CONDEL can be extended or adapted to data at hand and represents an "aggregator" approach to variant effect prediction. Condel scores can be derived for a limited set of specified mutations via an online web application. The Ensembl database provides a variation of position-specific CONDEL predictions that combine SIFT and Polyphen-2 for every possible amino acid substitution in all human proteins.

2.5. CHASM

Cancer-specific High-throughput Annotation of Somatic Mutations, or CHASM, is a computational method that identifies and prioritizes the missense mutations likely to enhance tumor cell proliferation (65). CHASM uses machine learning to classify putative "driver" cancer mutations as distinct from "passenger" mutations. Training the CHASM model employed *in silico* simulation to generate realistic "passenger" mutations, specifically modeled to represent variant context and genes that are observed in cancer settings. Multiple features of the variants, including their DNA and protein contexts, were then used to build a machine

learning approach that attempted to maximize the specificity of separating driver mutations from passenger mutations. CHASM represents a relatively specific algorithm focused not on "deleteriousness" but, rather, on the likelihood that an observed variant is a cancer "driver."

2.6. dbNSFP

Recognizing that applying all of the effect prediction tools available is potentially challenging (45), developed a database that aggregates predictions for *all* possible SNVs associated with coding changes (in Gencode gene models). With more than ten different prediction algorithms and extensive additional annotation, this database can be a useful one-stop-shop for adding annotations to variant datasets. The snpEff suite (described below) can be used in conjunction with dbNSFP to efficiently annotate SNPs with the potential to effect coding genes.

3. CLINICAL ACTIONABILITY

The ultimate goal for many of the abovementioned resources is to develop an individualized approach to the diagnosis, prevention, and treatment of cancer, or precision oncology. However, despite recent advances in HTS, determining the clinical relevance of experimentally observed cancer variants remains a challenge in the application of HTS in clinical practice. Difficulties in differentiating driver and passenger mutations, lack of standards and guidelines in reporting and interpretation of genomic variants, lack of clinical evidence in associating genomic variants to clinical outcome, lack of resources to disseminate clinical knowledge to the cancer community, and the precise definition of actionability have been reported to contribute to the bottleneck (68–71). Comprehensive resources linking experimentally determined cancer variants and clinical actionability have been developed to address some of these challenges and address various aspects of translating research results into clinical valuable information to support clinical decisions in precision oncology (see **Table 3**). In recognition of the fact that central curation of information regarding actionability is extremely challenging, several of the resources below use crowdsourcing as a means of gathering updates and enhancing curation efforts. In addition to a web interface, some tools provide additional access via API, mobile app, and/or social media tagging to facilitate dissemination of information and enhance accessibility. While some of these tools share similar functions, in the section below, we highlight distinct features and capabilities for a representative set of resources that might be used as a "starter" set for clinical annotation of variants.

The myvariant.info database is one of the newest and attempts to provide a "one-stop-shop" for variants. It is included in this section because it has recently incorporated the CIViC and Cancer Genome Interpreter databases. In addition, it provides annotations for SNVs from multiple other data sources (a growing list, so see the site for updates) and aggregates functional annotations for variants present in its database, making it a good all-around tool for cancer variant annotation. It is available as a performant web API only at this time.

Clinical Interpretation of Variants in Cancer (CIViC) is an open access and open source platform for community-driven curation and interpretation of cancer variants. It is based on a crowdsourcing model where individuals in the community can contribute to produce a centralized knowledge base with the goal of disseminating knowledge and encouraging active discussion. Users, including patients, patient advocates, clinicians, and researchers, can participate, along with community editors, in various stages of interpreting the clinical significance of cancer variants using standards and guidelines developed by community experts (68, 72).

The Drug Gene Interaction Database (DGIdb) is an open source and open access platform for gene and drug annotation for known interaction and potential druggability. Users can cross-reference genes of interest and drugs against up to 15 sources and in functionally classified gene categories (73, 74). Cancer Genome Interpreter (CGI) identifies mutational events that are biomarkers of drug response or interact with known chemical compounds (75). PharmGKB is a pharmacogenomic resource for building clinical implementation and interpretation based on annotating, integrating, and aggregating knowledge extracted from research-level publications. It provides scored clinical annotation, prescription annotation (drug dosing, prescribing information), as well as pharmacokinetics/pharmacodynamics (PK/PD) annotation, with primary literature reference.

TABLE 3 | In a clinical setting, these databases are the most relevant, as they are maintained to provide clinically actionable and curated content.

Resource	URL	Reference	Crowd-sourcing used	Bulk access
myvariant.info[a]	http://myvariant.info/	(46)	Yes	API[a]
CIViC[a]	https://civic.genome.wustl.edu/home	(72)	Yes	API, Download
DGIdb[a]	http://dgidb.genome.wustl.edu/	(73, 74)	Yes	API, Download
Cancer Genome Interpreter[a]	https://www.cancergenomeinterpreter.org/home	(75)	Yes	API
OncoKb[a]	http://oncokb.org/	(76)		API
Cancer Driver Log	https://candl.osu.edu/	(77)	Yes	Download
Clinical Knowledge Base	https://www.jax.org/clinical-genomics/clinical-offerings/ckb			
My Cancer Genome	http://www.mycancergenome.org	(78)	Yes	(licensed) API
Personalized Cancer Therapy	https://pct.mdanderson.org		Account required	
PharmGKB	https://www.pharmgkb.org/	(79)	Yes	Download
Precision Medicine Knowledge Base (Beta)	https://pmkb.weill.cornell.edu/	(80)	Yes	

While evalutation of each database by both clinical and informatics team members, databases marked with "[a]" are maintained, recently (or continuously) updated, and curated. The myvariant.info database includes both CiVIC and Cancer Genome Interpreter data. The last column in the table notes bulk access approaches as these are relevant when including databases in an annotation pipeline or automated report.

OncoKb contains information on the clinical implication of specific genetic alterations in cancer. Each variant is annotation from multiple sources and scored using Levels of Evidence ranging from Level 1, which includes FDA-approved biomarker predictive of response to an FDA-approved drug, to Level 2, which includes variants for which an FDA-approved or standard of care treatment is available, Level 3 and Level 4 contain variants with investigational and hypothetical therapeutic implications, respectively. A similarly structured scoring system is available for indicating therapeutic implications for variants associated with resistance (76). Cancer Driver Log (CanDL), an expert-curated database for potential driver mutations in cancer, employs a similar four-level scoring system based on FDA approval, clinical, pre-clinical, and experimental functional evidence (77).

MyCancerGenome (MCG) is a knowledge resource highlighting the implication of tumor mutation on cancer care. It allows users to access its content via a mobile app and provide patient-focused information. Patients can access a database entitled DNA-mutation Inventory to Refine and Enhance Cancer Treatment (DIRECT) for Epidermal Growth Factor Receptor (EGFR) mutation for non-small cell lung cancer (NSCLC). Personalized Cancer Therapy (PCT) at the MD Anderson Cancer Center is a resource for clinical response associated with cancer variants and aims to facilitate patient involvement in biomarker-related clinical trials. Drug effectiveness is associated with a specific biomarker and scored based on prospective clinical study as well as Food and Drug Administration (FDA) approval.

4. TOOLS FOR MANIPULATING VARIANT DATASETS

Processing sequence data with the goal of determining variants (somatic or germline) often end with a file in Variant Call Format (VCF format), a loose, self-describing data standard describing variants along a genome, associated statistical and numeric metrics for each variant, and information integrated from data resources such as those described in the preceding sections (81). An ecosystem of tools, listed in **Table 4**, has been developed for basic transformations, manipulations, merge operations, and for adding transcript, protein, and higher-level functional annotations to variants in a VCF file. The vt and bcftools software suites perform operations such as slicing by genomic coordinate, data compression, and, importantly variant normalization, rendering variants more readily comparable across resources. Annovar (82, 83) and the SnpEff suite (84) add annotations relative to gene annotations, including information about transcript and protein-coding changes. The Ensembl Variant Effect Predictor (VEP) utilizes Ensembl gene models to annotate variants in gene context and offers an interesting plugin architecture that supports adding variant information from resources in (**Table 1**) (85). Recently, several software developers of variant annotation tools have developed a standard for reporting gene-centric annotations that has simplified post-processing of variants after annotation. Finally, tools such as Vcfanno (86) have been developed that can flexibly add fields to variants in a VCF file based on relatively sophisticated logic and data transformations, reducing the number of tools required to bring a new data resource into the annotation pipeline.

TABLE 4 | Software tools for manipulating and adding annotations to variant datasets.

Software	URL	Reference
vt	http://genome.sph.umich.edu/wiki/Vt	(87)
bcftools	http://www.htslib.org/download/	(88)
ANNOVAR	http://annovar.openbioinformatics.org/en/latest/	(83)
Ensembl Variant Effect Predictor (VEP)	http://www.ensembl.org/vep	(85)
SnpEff	http://snpeff.sourceforge.net/	(84)
Oncotator	https://portals.broadinstitute.org/oncotator/	(89)
vcfanno	https://github.com/brentp/vcfanno	(86)

*Variant calling produces a list of observed variants. The tools in this table are useful for adding biological interpretation and for annotating the variants with information from resources in **Tables 1–3**.*

5. DISCUSSION

5.1. Pragmatic Details

Despite advanced toolsets for manipulating variant files and increasing adoption available standard formats, practical pitfalls and challenges remain to the basic manipulation of variant datasets. Some data resources are available in multiple formats and not all formats contain identical information. Matching variants between resources and observed variants can be challenging, as some variants can be represented validly in multiple forms. Ideally, variants are cataloged with clarity with respect to a reference genome and, whenever possible, using HGVS nomenclature (90). In spite of increasing awareness and uptake of HGVS standard nomenclature, the critical step of matching variants across tools and databases in assessing clinical significance is still hampered by inconsistencies across tools and databases (91). Particularly, when handling clinical samples, an information system that provides results from multiple resources when assessing novel variants, incorporates *in silico* controls when adding or updating data resources (to avoid introducing errors), and adheres to HGVS nomenclature wherever possible in data processing pipelines can increase the likelihood of discovering potentially relevant variants.

5.2. Where to Start?

This review is meant to be comprehensive, so the reader might wonder "Where do we start?." While it is difficult to make hard-and-fast recommendations about what resources, tools, and databases are "the best" given the lack of gold-standard datasets on which to base such evalutations, annotations in **Tables 1–3** are meant to provide context for prioritization. The context for sequencing (clinical or not, targeted mutations, trial setting, or novel variant and biomarker discovery) will also drive annotation pipeline development. Not all data resources need to be added simultaneously if developing a pipeline for annotating cancer variants for precision oncology applications. In a clinical setting, targeting the reporting workflow and working with clinicians to understand the most relevant annotations is the most efficient

approach to determining relevant resources for annotation. Developing a modular informatics pipeline, perhaps using a computational workflow framework (https://github.com/pditommaso/awesome-pipeline) that can be easily extended and re-run on previously annotated data is helpful to keep pace with the rapidly changing and growing collection of annotation resources. Newer aggregation resources such as myvariant.info offer a wholistic solution (annotation, catalog, and clinical actionability), but with some risk of "lossiness" with respect to the primary resources contained within.

Finally, given the rapid pace of new development in this space, we have established a crowd-sourced list of cancer variant resources for precision medicine available at https://github.com/seandavi/awesome-cancer-variant-databases.

5.3. Conclusion

Robust sequencing technologies and increasingly reliable bioinformatics pipelines, combined with parallel development of therapeutics and diagnostics has bolstered the field of precision genomic oncology. However, the sheer number of resources available that can inform the interpretation of small variants is staggering, except for the very few variants with well-established clinical relevance or an associated targeted therapy. This review has highlighted a number of important data resources individually. For other variants, data integration remains a significant hurdle to the rapid turnaround required to apply HTS in a clinical context. Expert panel review (the molecular tumor board) has been effective for some groups (13, 92, 93) while other groups have adopted a protocol-based approach (94). Even when molecularly targetable lesions are identified, barriers to delivering therapy have been observed, limiting the impact of precision genomic oncology in some settings (95). Not covered in this review is the increasing utility of HTS in the burgeoning field of immunotherapy, where early efforts to predict response based on HTS results have been promising (19, 96, 97).

Some interesting trends are evident in the databases and resources presented in this review that highlight the overarching trends in delivering precision medicine. First is the sheer volume and rapid growth of numbers of observations to learn about the spectrum of variation cancer and normal genomes. Projects such as GnomAD, COSMIC, and other data sharing efforts enhance precision by cataloging rare variants as well as precise estimates of the frequencies of common variants. Second is the use of crowd-sourcing to produce rich clinical annotation (e.g., CiVIC) in response to the need for intensive human interaction to interpret the clinical impact of a variant or its relationship to potential medical intervention. On the other hand, with volumes of data ever-increasing, machine learning techniques drive many of the most commonly used approaches for assigning scores for impact of observed variants. As well-annotated datasets and variant catalogs grow, application of machine learning will become both more common and more powerful.

While significant progress has been made in applying technology to precision oncology, cancer arises in an individual after a typically complex and incompletely understood set of oncogenic events that are increasingly observable at the molecular level. Progress in cancer prevention, early detection, diagnosis, prognosis, and treatment is increasingly driven by insight gained through the analysis and interpretation of large genomic, proteomic, and pharmacological knowledge bases. Reductionist approaches to cancer biology can achieve only limited success in understanding cancer biology and improving therapy. Cancer is a disease associated with disruption of normal cellular circuitry and processes that leads to abnormal or uncontrolled proliferative growth, characterized by a complex spectrum of biochemical alterations that affects biological processes at multiple scales from the molecular activity and cellular homeostasis to intercellular and inter-tissue signaling. The cancer research community has made great strides in measuring the oncogenic events that lead to the development of cancer and therapy resistance. Because of the complexity inherent in protein networks, intercellular signaling, cellular heterogeneity, and the dynamic nature of cancer, future progress will require a more wholistic approach to precision oncology, including multiscale systems and modeling approaches that address the interrelatedness of the biological processes underlying cancer.

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Therapy for Cancer: Strategy of Combining Anti-Angiogenic and Target Therapies

Valentina Comunanza[1,2] *and Federico Bussolino*[1,2*]

[1] Department of Oncology, University of Torino, Candiolo, Italy, [2] Candiolo Cancer Institute FPO-IRCCS, Candiolo, Italy

The concept that blood supply is required and necessary for cancer growth and spreading is intuitive and was firstly formalized by Judah Folkman in 1971, when he demonstrated that cancer cells release molecules able to promote the proliferation of endothelial cells and the formation of new vessels. This seminal result has initiated one of the most fascinating story of the medicine, which is offering a window of opportunity for cancer treatment based on the use of molecules inhibiting tumor angiogenesis and in particular vascular-endothelial growth factor (VEGF), which is the master gene in vasculature formation and is the commonest target of anti-angiogenic regimens. However, the clinical results are far from the remarkable successes obtained in pre-clinical models. The reasons of this discrepancy have been partially understood and well addressed in many reviews (Bergers and Hanahan, 2008; Bottsford-Miller et al., 2012; El-Kenawi and El-Remessy, 2013; Wang et al., 2015; Jayson et al., 2016). At present anti-angiogenic regimens are not used as single treatments but associated with standard chemotherapies. Based on emerging knowledge of the biology of VEGF, here we sustain the hypothesis of the efficacy of a dual approach based on targeting pro-angiogenic pathways and other druggable targets such as mutated oncogenes or the immune system.

Keywords: cancer, VEGF, angiogenesis, target therapy, resistance

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***Correspondence:**
Federico Bussolino
federico.bussolino@unito.it

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VEGF-TARGETED ANTI-ANGIOGENIC THERAPY

During tumor progression, some clones experience the "angiogenic switch" by interrupting the balance between angiogenesis inducers and inhibitors and show pro-angiogenic phenotype. As a result, initial lesions or dormant metastases become more aggressive (Hanahan and Folkman, 1996; Wicki and Christofori, 2008). Angiogenesis inhibitors were postulated as anticancer drugs in the early 1970s (Folkman, 1971). Of all identified molecules that lead the blood vessel formation, VEGFA appears the main molecular driver of tumor angiogenesis. Indeed VEGFA is overexpressed in the majority of solid tumors and for this reason is the dominant target for antiangiogenic drugs (Carmeliet and Jain, 2000; Ferrara, 2002; Kerbel, 2008). VEGF/platelet-derived growth factor (PDGF) protein family is characterized by the presence of a structural motif with eight conserved cysteine residues forming the typical cystine-knot structure and include a wide range of angiogenic inducers: VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and placental growth factor (PLGF). The main signaling tyrosine kinase receptor (TKR) is VEGF-receptor, VEGFR2 (also known as KDR) (Ferrara and Kerbel, 2005). Two other VEGFRs are VEGFR1 and VEGFR3 (**Figure 1**). In embryo as well as in solid tumors VEGF expression is primarily stimulated by hypoxia and VEGFA

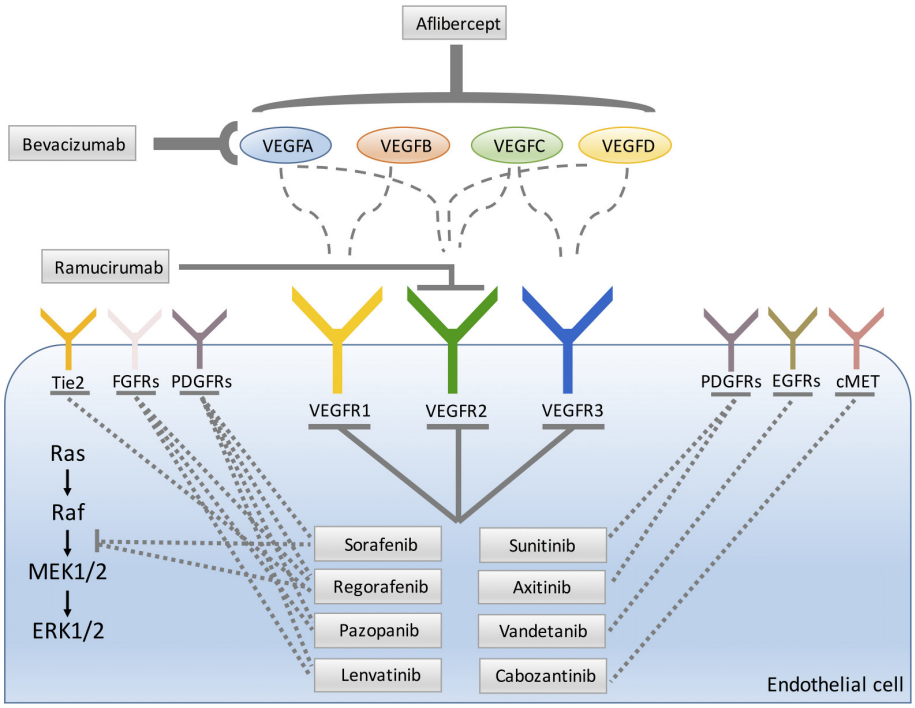

FIGURE 1 | Main molecular targets of anti-angiogenic drugs approved for patients treatment.

transcription is promoted by hypoxia-inducible factor-1α (HIF1α) and −2α (HIF2α) that sense the reduced pO_2 (Semenza, 2009).

Besides favoring the tumor feed, a consequence of "angiogenic switch" is the abnormality of the vessel architecture and defects of microcirculation rheology. The aberrant amounts of angiogenic inducers accelerate the proliferation of endothelial cells with a reduced time frame to allow the whole capillary maturation. As consequence, capillaries are tortuous, irregularly fenestrated with reduced pericyte coverage and leaky. These morphological aberrations induce the increase of interstitial pressure with the decrease of convective transport of small molecules including chemotherapeutics (Nagy et al., 2006; Jain, 2014).

The rationale proposed by Dr. Folkman to exploit anti-angiogenic compounds in clinical settings was to starve cancer and induce its dormancy. Currently, preclinical data suggest that a drastic pruning of tumor vasculature results in a selection of more aggressive cancer clones, which sustain disease progression (Ebos et al., 2009; Pàez-Ribes et al., 2009; Maione et al., 2012). Of interest, some studies failed to document such effects in other preclinical models (Singh et al., 2012).

However, before reaching the whole collapse of vascular bed, VEGF pathway blockade is characterized by an early and transient phase in which vessels assume normal shape and function (Folkman, 2006; Carmeliet and Jain, 2011; Goel et al., 2011). This normalization is characterized by rescue of the balance between inhibitors and inducers of angiogenesis, reduction of leakage and interstitial pressure, improvement of tumor perfusion and oxygenation, and drug delivery. This effect is potentially sensitizing for radiotherapy and increases tumor exposure to cytotoxic chemotherapy (Jain, 2005, 2014).

The degree of vascular normalization correlated with increased survival in glioblastoma multiforme (GBM) preclinical models (Kamoun et al., 2009) and patients (Sorensen et al., 2012; Batchelor et al., 2013). Vascular normalization can also improve trafficking of immune effector cells into tumor microenvironment and prolongs the survival of tumor-bearing mice receiving active immunotherapy (Huang et al., 2013; Jain, 2014; Kwilas et al., 2015).

Anti-Angiogenic Regimens in Advanced Cancers

In general, the inhibition of tumor angiogenesis can be reached by the withdrawal of pro-angiogenic molecules or by inhibiting the signaling pathways triggered by these molecules. Most of angiogenesis inhibitors approved in human cancers targets VEGFA and its VEGFRs pro-angiogenic–mediated signals. The pioneer of angiogenesis inhibitors is the VEGFA-targeted monoclonal antibody bevacizumab (Ferrara et al., 2004; Kerbel, 2008; **Figure 1**). In contrast to most preclinical studies, monotherapy with bevacizumab failed to increase patients' overall survival (OS) (Jain, 2005), but in combination with chemotherapy it can extend progression free survival (PFS) and/or OS in several cancer types including metastatic colorectal cancer (mCRC) (Hurwitz et al., 2004; Giantonio et al., 2007; Cunningham et al., 2013) and recurrent GBM (Vredenburgh et al., 2007; Friedman et al., 2009). Based on the results of these

trials, bevacizumab was approved for the treatment of patients with late stage CRC, non-small cell lung cancer (NSCLC), ovarian cancer, metastatic cervical cancer, metastatic renal cell carcinoma (RCC), and GBM, only when given in combination with chemotherapy (**Table 1**). As concern breast cancer, Food and Drug Administration (FDA) approved the combination of bevacizumab with paclitaxel for the treatment of human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (Miller et al., 2007). However, three further phase III trials, failed to confirm the efficacy of the association of bevacizumab with chemotherapy in metastatic breast cancer (Miles et al., 2010; Brufsky et al., 2011; Robert et al., 2011a) resulting in the withdrawal of approval by FDA.

Aflibercept, the "VEGF-trap," is a fusion protein engineered by joining the second Ig-like domain of VEGFR1 and the third Ig-like domain of VEGFR2 to a human IgG1 Fc-fragment (Holash et al., 2002). This soluble decoy receptor shows one-to-one high-affinity binding to all isoforms of VEGF and PLGF (**Figure 1**). Clinical randomized phase III trials using aflibercept were performed for several solid cancers (Ciombor et al., 2013) and the addition of this compound to standard therapies lengthened PFS and OS in mCRC patients who progressed on bevacizumab therapy (Van Cutsem et al., 2012a). FDA approved aflibercept in combination with leucovorin, 5-fluorouracil and irinotecan (FOLFIRI) for treating patients after progression with oxaliplatin-containing regimen (Ciombor et al., 2013; **Table 1**). Furthermore, promising experimental models propose aflibercept as a promising candidate to treat, hepatocarcinoma (HCC) (Torimura et al., 2016), a highly vascular tumor with the development of neoarteries in parallel with tumor growth.

Ramucirumab is a monoclonal antibody that binds the extracellular domain of VEGFR2 and interferes with VEGF binding to its receptor. FDA and EMA (European Medicines Agency) approved this compound either as single agent or in association with paclitaxel in subjects affected by metastatic gastric and gastroesophageal junction cancer after progression on fluoropyrimidine or platinum containing protocols (Fuchs et al., 2014; Wilke et al., 2014; **Figure 1**). Subsequently, ramucirumab was approved for the second-line treatment of NSCLC with active disease progression or after platinum-based therapy and for the treatment of mCRC in combination with FOLFIRI in patients whose disease was insensitive to bevacizumab, oxaliplatin and fluoropyrimidine (**Table 1**).

A number of small molecules inhibiting the TK activity of VEGFR, principally (VEGFR2) have been approved as single therapies (**Figure 1**). Among this class of agents, the pioneer drugs have been sorafenib and sunitinib. Sorafenib is a multikinase inhibitor that targets VEGFR1-3, PDGFRβ, FLT-3, Ret, c-kit, RAF-1, BRAF (Wilhelm et al., 2004). Due to its anti-proliferative, apoptotic, anti-angiogenic and anti-fibrotic effects, sorafenib is a compound with a potent antitumoral activity. Sorafenib is currently the only approved systemic treatment for HCC (Llovet et al., 2008) and several reports have stressed the role of VEGF in the vascularization process of this neoplasia (Miura et al., 1997). Sorafenib has also been approved for the treatment of advanced renal cell carcinoma (RCC) and thyroid cancers (**Table 1**). The multi-targeted kinase inhibitor sunitinib (VEGFRs, PDGFRs, FLT3, CSFF1R) has been approved for RCC and pancreatic neuroendocrine tumors (**Table 1**).

TABLE 1 | Approved VEGF-targeted therapy for oncology.

Drug	Brand name	Mechanism	Indications
Bevacizumab	Avastin (Genentech)	Monoclonal anti-VEGF antibody	CRC; NSCLC; RCC; GBM; epithelial ovarian cancer; fallopian tube cancer; primary peritoneal cancer; cervical cancer
Aflibercept	Zaltrap (Sanofi and Regeneron Pharmaceuticals)	Recombinant fusion VEGF protein	CRC
Ramucirumab	Cyramza (Eli Lilly and Company)	Monoclonal anti-VEGFR2 antibody	CRC ; NSCLC; gastric or gastroesophageal junction adenocarcinoma
Sorafenib	Nexavar (Bayer)	Multi-TKI (VEGFRs, PDGFRs, RAF, KIT, FLT3, RET)	RCC, HCC, thyroid cancer
Sunitinib	Sutent (Pfizer)	Multi-TKI (VEGFRs, PDGFRs, FLT3, CSF1R, RET)	RCC, pancreatic neuroendocrine tumors, gastrointestinal stromal tumors
Regorafenib	Stivarga (Bayer)	Multi-TKI (VEGFRs, PDGFRs, FGFRs, TIE2, KIT, RET, RAF)	GIST, CRC, HCC
Pazopanib	Votrient (GlaxoSmithKline)	Multi-TKI (VEGFRs, PDGFRs, FGFR1, c-Kit)	RCC, soft tissue sarcoma
Axitinib	Inlyta (Pfizer)	Multi-TKI (VEGFRs, PDGFRs, c-Kit)	RCC
Vandetanib	Caprelsa (AstraZeneca)	Multi-TKI (VEGFRs, EGFR, RET)	medullary thyroid cancer
Lenvatinib	Lenvima (Eisai)	Multi-TKI (VEGFRs, FGFRs, PDGFRa , RET, c-Kit)	thyroid cancer, RCC
Cabozantinib	Cometriq (Exelixis)/Cabometyx (Exelixis)	Multi-TKI (VEGFRs, cMet, AXL)	medullary thyroid cancer, RCC

CSFR1, colony stimulating factor 1 receptor; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; FLT3, Fms-like tyrosine kinase 3; GBM, glioblastoma multiforme; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma; KIT, stem cell factor receptor; MET, hepatocyte growth factor receptor; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; RAF, rapidly accelerated fibrosarcoma; RCC, renal cell carcinoma; RET, rearranged during transfection; VEGFR, vascular endothelial growth factor receptor.

Anti-angiogenic therapies currently approved by the US Food and Drug Administration (FDA) for the treatment of malignancies (July 2017).

For reference see http://cancer.gov.

Subsequently, other agents were developed with similar targets but are characterized by better toxicity profiles. This second-generation of multi-kinases inhibitors have improved target affinity and less off target effects thus allowing lower concentrations of active drugs to be administered with significant activity. Regorafenib belongs to this second-generation of oral multikinase inhibitors that blocks the activity of several kinases, including those involved in the regulation of tumor angiogenesis (VEGFR1-3 and TIE2), oncogenesis (KIT, RET, RAF1, BRAF and $BRAF^{V600E}$) and the tumor microenvironment (PDGFRβ and FGFR). Moreover, it has been recently shown that regorafenib also exerts anti-metastatic activity because of its capability to inhibit epithelial-mesenchymal transition (Fan et al., 2016). This drug represents a significant improvement over the first-generation of TKI due to its higher specific activity leading to greater pharmacology potency (Wilhelm et al., 2011). Recently, a phase III study showed that regorafenib extended OS and PFS in mCRC patients previously progressed on standard therapies (Grothey et al., 2013). Regorafenib is now approved for the treatment of mCRC and gastrointestinal stromal tumors (Demetri et al., 2013; **Table 1**).

Among the second-generation multi-kinases class of inhibitors also pazopanib (Gupta and Spiess, 2013), cabozanitinib (Singh et al., 2017), lenvatinib (Fala, 2015), axitinib (Tyler, 2012), and vandetanib (Degrauwe et al., 2012) have been approved as single therapies in specific indications (**Table 1**).

Recently, based on the result of the phase III LUME-Lung 1 trial (Reck et al., 2014) EMA, but not FDA, approved the use of nintedanib, an oral multi-kinases inhibitor, targeting VEGFR1-3, FRGFR1-3, PDGFRα-β, RET, FLT3, and Src family kinases, combined with docetaxel for the second-line treatment of NSCLC (Lazzari et al., 2017). Moreover, phase II LUME-Meso trial suggested an improvement of PFS of malignant pleural mesothelioma treated with nintedanib in combination with standard treatments (Scagliotti et al., 2016).

Anti-Angiogenic Regimens in Adjuvant Settings

The use of VEGF pathway inhibitors has been started to investigate in phase II and III trials in adjuvant (post-surgical) and neoadjuvant (pre-surgical) settings. Anti-angiogenic agents are used in the adjuvant setting according to the concept that halting angiogenesis after the removal of primary tumor may prevent local relapse micrometastasis spreading tumors. However further clinical and preclinical findings raise doubts on the efficacy of VEGF pathway inhibitors in this setting (Ebos and Kerbel, 2011). Actually, many phase III adjuvant trials with VEGF-targeted therapy failed in CRC, breast cancer, RCC and HCC (de Gramont et al., 2012; Cameron et al., 2013). The reasons of these disappointing results are largely unknown. Probably the different biology of micrometastases from that of established metastatic disease may alter the response to anti-angiogenic agents (Vasudev and Reynolds, 2014).

In neoadjuvant settings, antiangiogenic treatments are used to downsized a tumor, resulting in potentially surgically treatable lesion. Furthermore, it might be used to reduce the risk of local relapse or metastasis. Interestingly the use of bevacizumab (with chemotherapy) in an neoadjuvant setting showed a pathological complete response in breast tumors (Bear et al., 2012; von Minckwitz et al., 2012; Earl et al., 2015; Sikov et al., 2015). Of interest, the efficacy of bevacizumab in promoting vascular normalization in breast tumors correlated with a high baseline microvessel density (MVD), suggesting that basal MVD is a potential biomarker of response to bevacizumab in breast cancer (Tolaney et al., 2015).

The Combination of Anti-Angiogenic Regimens with Chemotherapy

As reported above, anti-angiogenic regimens targeting the excess of angiogenic inducers (e.g., bevacizumab or aflibercept) show clinical benefits when associated with cytotoxic therapies (chemotherapy or radiation). Two different observations sustain this rationale. First, this combined strategy can destroy two separate compartments of tumors: cancer cells and endothelial cells (Teicher, 1996). Furthermore, there is a possible synergistic effect of chemotherapy on endothelial compartment by inhibiting endothelial cell cycle. Metronomic chemotherapy is based on this premise and aims at controlling tumor growth by the frequent administration of conventional chemotherapeutic agents at very low doses to target activated endothelial cells in tumors as well as cancer cells, the advantages of which include minimal adverse effects and a rare chance of developing acquired drug resistance (Kerbel, 2015). Second, the vascular normalizing effects of anti-angiogenic regimen modifies the pharmacokinetics parameters of small molecules and favors the delivery of cytotoxic drugs (Zhou et al., 2008; Emblem et al., 2013).

In contrast to anti-angiogenic compounds neutralizing the excess of angiogenic inducers, TKIs do not show any clinical improvement when administered with standard therapies. For instance, attempts to combine anti-angiogenic TKIs with chemotherapy did not improve PFS in mCRC (Carrato et al., 2013) and metastatic breast cancer (Robert et al., 2011b). Indeed, VEGF receptor TKIs exhibit single-agent activity and are effective as monotherapy, while show toxicity in combination with chemotherapy (Jain et al., 2006).

MECHANISMS OF RESISTANCE TO ANTI-ANGIOGENIC REGIMENS

Despite the partial clinical success VEGF-targeted therapies in cancer, some refractory patients do not respond to the treatments (intrinsic resistance) or undergo to acquired resistance after transitory benefits (Bergers and Hanahan, 2008). The extent of refractoriness differs for VEGF blockers and for different cancer types and metastatic settings. Intrinsic and acquired modes of resistance recognize partially overlapping mechanisms, but on the clinical point of view the later represents the most difficult obstacle to achieve better clinical results with anti-angiogenic regimens.

Here we summarize the principal cellular and molecular mechanisms leading to the cancer resistance to anti-angiogenic compounds.

The Vascular Features of the Tumors

The development of anti-angiogenic strategies started before the genomic revolution signed by the first description of human genome and was largely based on a reductionist perspectives and approaches. VEGF was identified as the master tumor angiogenic inducer and "sprouting angiogenesis" (i.e., the formation of capillaries from pre-existing vessels by endothelial sprouting triggered by angiogenic inducers and followed by formation of endothelial tubes, which undergo maturation by pericyte recruitment and extracellular matrix remodeling) as the almost unique mode to sustain the tumor vascularization (Bussolino et al., 1997). The ability of a cancer clone to trigger an angiogenic response is strictly dependent on its pattern of genomic alterations (Rak et al., 1995; Arbiser, 2004), which evolve along the time of the disease and under the pressure exerted by pharmacological treatments. This situation can be exacerbated by the recent genomic findings revealing evidence of branched evolution, wherein tumors consist of multiple distinct subclones that share a common ancestor but differ in terms of subtle or deep genomic alterations that occur later in the evolution of the cancer (Swanton and Govindan, 2016). Such subclones may be intermixed within one tumor sample or regionally separated within a primary tumor, between primary and metastatic sites, or between metastatic sites (Abbosh et al., 2017; Jamal-Hanjani et al., 2017).

Moreover, communication circuits between cancer and stroma cells result in the production a plethora of angiogenic inducers that can support vascular growth and fitness in the presence blockers of VEGF action. This scenario can precede the use of anti-VEGF therapy and explain the intrinsic resistance or be triggered by VEGF inhibitors resulting in a mode of adaptive resistance (Jayson et al., 2016).

Pre-clinical studies identified numerous candidates that can substitute VEGF in sustaining tumor angiogenesis and include angiopoietins (Ang), ephrins, fibroblast growth factor-1 (FGF1) and−2 (FGF2) (Casanovas et al., 2005), prokineticin-1 (Bv8) (Shojaei et al., 2007b), hepatocyte growth factor (HGF) (Shojaei et al., 2010; Cascone et al., 2017), IL-8 (Huang et al., 2010), platelet-derived growth factor C (PDGFC) (Crawford et al., 2009), VEGFC (Li et al., 2014), and PLGF (Fischer et al., 2007). Most of these studies also show that co-targeting of VEGF and the candidate factor improves therapeutic response. In support to this, clinical evidence show that circulating levels of pro-angiogenic factors, including FGF2, HGF, PLGF, and PDGF can become elevated in patients related to the development of acquired resistance to VEGF blockade (Kopetz et al., 2010).

A more intricate connection exists between the resistance to anti-VEGF therapies and Dll4/Notch axis. In sprouting angiogenesis the expression of Dll4 and Notch are increased by VEGFA and counteract the its proangiogenic effect (Thurston et al., 2007). Interestingly, up-regulation of Dll4 induces resistance to bevacizumab in GBM preclinical models, and is in turn overcame by Notch inhibition by γ-secretase inhibitors (Li et al., 2011). Moreover, it has been also reported that high Dll4 expression is predictive of favorable clinical response to anti-VEGF regimen in ovarian cancer (Hu et al., 2011).

VEGF blockers and more in general all compounds devised to interfere with an angiogenic inducer halt sprouting angiogenesis. However, established evidences indicate that the tumor mass can be vascularized by vessel co-option, a process wherewith tumor cells simply incorporate pre-existing capillaries from surrounding tissue (Holash et al., 1999). Recently, it has reported in metastases blood supply occurs by the non-angiogenic mechanism of vessel co-option (Donnem et al., 2013; Frentzas et al., 2016). The prevalence of vessel co-option in breast cancer and in liver metastasis of CRC (Frentzas et al., 2016) could explain why anti-angiogenic therapies were poorly effective in approaching metastatic breast cancer and showed a moderate efficacy in metastatic CRC.

Pre-clinical evidences support the role of vessel co-option in the onset of resistance to anti-angiogenic regimens in GBM (Rubenstein et al., 2000), HCC (Kuczynski et al., 2016), and in metastasis to lungs (Bridgeman et al., 2017), lymph nodes (Jeong et al., 2015), and liver (Frentzas et al., 2016). Adjuvant trials in thousands of patients with breast cancer and CRC (de Gramont et al., 2012; Cameron et al., 2013) have been negative probably because micrometastases co-opt existing vessels. Therefore, vessel co-option—mediated blood delivery to the growing tumors and metastases can contribute to both adaptive (e.g., in metastatic disease) and intrinsic resistance.

Besides vessel co-option other tumor vascularization mechanisms have been described and are not sustained by sprouting angiogenesis. They include vascular mimicry, in which tumor cells replace endothelial cells to form the capillary wall; tumor vasculogenesis, which is characterized by the recruitment of endothelial precursors from bone marrow and intussusceptive angiogenesis characterized the duplication of a pre-existing vessel by a splitting mechanism. However the clinical relevance of these mechanisms in mediating resistance to anti-angiogenic compounds remain unclear (Lyden et al., 2001; Semela et al., 2007; Kirschmann et al., 2012).

Finally some tumors but in particular pancreatic ductal adenocarcinomas can exhibit primary refractoriness, manifest as a tumor type that is poorly vascularized with a prominent fibrotic reaction and able to survive in adverse and most probably hypoxic conditions (Ryan et al., 2014). A similar circumstance can explain the lack of the effect of anti-angiogenic regimens in the treatment of prostate cancer (Taverna et al., 2013; Jayson et al., 2016).

Tumor Microenvironment

The features of the stroma cells (leucocytes, pericytes, and fibroblasts) in tumors can deeply influence the initial response to angiogenic-regimens as well as the establishment of acquired resistance.

A wide range of myeloid and lymphoid cells can dynamically visit solid tumors. The presence of M2 polarized macrophages or immature $Tie2^{+}$ monocytes can configure a precise circumstance rendering poorly effective the effects of VEGF blockers and specific myeloid-mediated circuits are activated by anti-VEGF therapies and trigger the onset of acquired resistance (Mantovani and Allavena, 2015). Of notice, refractoriness to antiangiogenic therapies, in GBM patients, is associated with higher numbers

of $CD68^+$ TAMs and $CD11b^+$ myeloid cells and the increase of these populations is associated with poor survival (Lu-Emerson et al., 2013).

In particular, a specific circuit eliciting the acquired resistance to anti-VEGF antibodies has been described and involves $CD11b^+/Gr1^+$myeloid cells and T_H17 lymphocytes, which represent a subset $CD4^+$ T cells producing IL-17. It is plausible to envisage that in response to VEGF removal more T_H17 are recruited and/or start to produce IL-17, which in turn activates the release of G-CSF from stroma cells (Chung et al., 2013). G-CSF is an angiogenic inducer (Bussolino et al., 1989) and is crucial for the mobilization and recruitment of $CD11b^{+/}Gr1^+$ population to the cancer microenvironment that are capable of promoting VEGF-independent tumorigenesis (Shojaei et al., 2007a). A second circuit entails $Ly6C^{lo}$ monocytes. Anti-VEGF therapy up-regulates CX3CL1 expression, which facilitates CX3CR1-dependent infiltration of $Ly6C^{lo}$ monocytes. These cells attract neutrophils via CXCL5, resulting in the formation of an immunosuppressive microenvironment with a reduction of cytotoxic T lymphocytes (Jung et al., 2017).

Pericytes are mesenchymal cells with contractile properties that patch the capillary outer surface and play a part in vascular physiology. Pericytes are recruited on vessels by PDGFB/PDGFRβ signaling both in physiologic and pathological conditions (Abramsson et al., 2003). In most tumor, vessels are surrounded by few pericytes, but in others a dense pericyte coat with thick basement membrane is present; such vessels are usually less sensitive to VEGF blockers (Bergers et al., 2003). Of interest, several anti-angiogenic TKIs clinical-approved are efficient blockers of both VEGF and PDGF receptors (e.g., sunitinib, sorafenib, pazopanib) and therefore may interfere in pericyte coverage.

Finally, cancer associated fibroblasts (CAFs) or fibrocytic cells recruited from bone marrow are cells that take part to the acquisition of resistance to VEGF-blockers by producing alternative pro-angiogenic substances (Crawford et al., 2009; Mitsuhashi et al., 2015).

Besides the cellular components of the stroma, the features of extracellular matrix can influence the refractoriness to anti-angiogenic therapies. It has been recently noted in human and mouse models of CRC liver metastatization that anti-VEGF therapy results in abnormal deposition of proteoglycans, in particular hyaluronic acid and sulfated glycosaminoglycans (Rahbari et al., 2016). Interestingly, the depletion of hyaluronic acid results in improved tumor perfusion and treatment efficacy in the mouse model of liver mCRC (Rahbari et al., 2016). These findings parallel the observation that anti-angiogenic therapy increases collagen expression, as a consequence of increased hypoxia, in murine models of pancreatic ductal adenocarcinoma (Aguilera et al., 2014) and HCC (Chen et al., 2014).

Adaption of Tumor Cells to Stressed Conditions

As discussed above, a negative consequence of a prolonged treatment with anti-angiogenic regimens is the reduced blood perfusion and metabolites' exchanges, which evolve in hypoxia and acidosis (Jain, 2014). Besides induction of epithelial-to-mesenchymal program that favors an invasive and metastatic tumor cell phenotype hypoxia is thought to select for tumor cells with cancer stem cell properties that might further mediate resistance to cytotoxic agents (Semenza, 2014). In mouse models, hypoxic stress promoted by short-term treatment with anti-VEGF molecules amplified tumor invasiveness and metastatic progression (Ebos et al., 2009; Loges et al., 2009; Pàez-Ribes et al., 2009). The rationale of this paradox is based on the effect on tumor metabolism exerted by the massive vessel pruning and the reduced blood perfusion. The generated hypoxic and acidotic stresses kill a huge amount of cancer cells, but few of them change their features to survive in these hostile conditions by adapting their metabolism, changing the expression of proton pumps, or through autophagy by activating AMP-kinase (Hu et al., 2012; Xu et al., 2013; Fais et al., 2014).

In particular, HIF1-mediated response favors the selection of more aggressive cancer clones (Semenza, 2009) and their metastatic phenotype (Maione et al., 2012) thus explaining the clinical observation that in some solid tumors anti-angiogenic molecules are effective in increasing PFS but they show a negligible effect on OS.

Furthermore, hypoxia favors an immunosuppressive microenvironment by reducing the activity of cytotoxic T cells and antigen-presenting cells and by skewing the polarization of TAMs toward protumorigenic and immunosuppressive M2 phenotype (Mantovani and Allavena, 2015). In HCC, it was demonstrated that increased hypoxia after sorafenib treatment induced $Gr1^+$ myeloid-derived suppressor cell recruitment (Chen et al., 2014).

Several pre-clinical studies report that VEGF-targeted therapy can promote increased tumor invasion and metastasis in a hypoxia-independent manner. It was demonstrated that VEGF suppresses HGF-dependent MET phosphorylation and tumor cell migration through the formation of a VEGFR2/MET heterocomplex. This mechanism could explain why VEGF blockade leads to a proinvasive phenotype in preclinical mouse models of GBM and in a subset of GBM patients treated with bevacizumab (Lu et al., 2012).

Biomarkers and Anti-Angiogenic Therapies

The clinical efficacy of an anti-angiogenic regimen is based on strategies mainly set-up to monitor the tumor cyto-reduction along chemotherapic and radiotherapic treatments. However, the effect of this kind of treatment does not necessarily induce a rapid reduction of tumor mass detectable by imaging approaches or by analyzing the decrease of plasmatic levels of molecules released by the tumor (specific proteins, microRNA, mutated DNAs). Similarly, the present knowledge does not allow predicting which cancer patient can really benefit of an anti-angiogenic treatment.

Huge efforts have been made to evaluate the potential value of circulating angiogenic inducers to address clinical strategies. However high plasmatic levels of VEGF do not predict a response to anti-VEGF/VEGFR2 compounds, and its fluctuation along the treatment is independent from the clinical efficacy (Kopetz et al., 2010). Recent studies have assessed the potential for other biomarkers detectable in plasma. In particular, the pretreatment

levels of soluble VEGFR1 inversely correlated with the outcome of either bevacizumab and TKIs because it acts as an endogenous VEGF trap (Meyerhardt et al., 2012; Zhu et al., 2013). Another postulated biomarker is the increased amount of CXCL12, which increased in subjects who escape to anti-angiogenic regimens (Zhu et al., 2009; Batchelor et al., 2010) while low amount of IL-8 at the baseline seems to predict a poor response to bevacizumab treatment in HCC (Boige et al., 2012). Conversely, low pre-treatment levels of Ang-2 were associated with a prolonged PFS in CRC treated with bevacizumab (Goede et al., 2010). Many other works showed an increase of angiogenic molecules along anti-angiogenic regimens and in particular bevacizumab, suggesting that the VEGF removal can trigger the activation of alternative pathways sustaining vascularization, reviewed in Lambrechts et al. (2013).

An emerging diagnostic area still not investigated in anti-angiogenic regimens is represented by circulating exosomes and their cargos (Wang et al., 2016), including microRNA that are promising markers in oncology (Lin and Gregory, 2015).

A second investigative area is the presence in primary tumors of molecules or vascular features, which can predict the response to angiogenesis inhibitors. Generally speaking, many data have been provided such as microvessel density and the expression of pro-angiogenic molecules (VEGFs, VEGFRs, HGF, PDGFs, chemokines, and Ang) but the results are largely contradictory and poorly robust in term of clinical analysis (Lambrechts et al., 2013). In this context one of the more promising result is the correlation between low level of neuropilin-1 expressed in a large cohort of gastric cancers and the prolonged OS after bevacizumab treatment (Van Cutsem et al., 2012b).

Tumors release a plethora of soluble molecules that have a major impact on the biology of bone marrow. Besides modifying the differentiation and the mobilization in particular of myeloid cells, these molecules can promote the mobilization of endothelial precursors. In particular it has been reported that VEGFA or PLGF released by tumor, through a mechanism dependent on metalloproteinase-9 and soluble Kit ligand, increase the number of these cells in bloodstream, while CXCL12 and CXCR4 receptor favor their retention in perivascular site of injured issues (Kopp et al., 2006). The preclinical observation that the number of circulation endothelial precursors was increased by vascular disrupting molecules (Shaked, 2006), many studies focused on the possibility that these cells could be used to monitor or predict the efficacy of anti-angiogenic drugs. Besides the lack of a solid consensus on their phenotype (Ingram et al., 2005) the clinical data on this approach in clinical oncology are conflicting (Bertolini et al., 2006). For instance anti-angiogenic treatment reduces circulating endothelial cells (Dellapasqua et al., 2008), while metronomic therapy shows an opposite effect (Mancuso et al., 2006).

A further promising area is the role exerted by specific single nucleotide polymorphisms (SNPs) of candidate genes to stratify responder and non-responder patients to anti-angiogenic regimen associated with standard therapies. VEGFR1 rs9582036 associated with an improvement of PFS and OS in patients with metastatic pancreatic adenocarcinoma treated with bevacizumab associated with chemotherapy. On the contrary in renal-clear carcinoma VEGFR1 rs7993418 correlated with PFS but not OS in the bevacizumab group (Lambrechts et al., 2012). Another example was reported in metastatic CRC where VEGFA rs833061 and VEGFR1 rs9513070 respectively associated with the objective response rate and the OS in subjects treated with cytotoxic chemotherapy plus bevacizumab (Sohn et al., 2014). Analysis of genetic variants of other angiogenic-related genes in breast cancer using neoadjuvant bevacizumab in combination with chemotherapy compared to chemotherapy alone showed a correlation between specific SNPs in term of pathologic complete response but not in OS (Makhoul et al., 2017).

In recent years, dynamic contrast-enhanced (DCE)-MRI, which enable non-invasive quantification of microvascular structure and function in tumors, has been extensively evaluated in clinical trials as a biomarker for predicting tumor vascular response to anti-angiogenic treatments (Morotti et al., 2017). VEGF blockade is believed to reduce tumor vascular permeability and perfusion. Significant reductions in capillary permeability have been observed in different studies of bevacizumab and TKI in monotherapy or combination with cytotoxic agents (O'Connor et al., 2012). More recently a further exploitation of DCE-MRI termed vessel architectural imaging allowed the vessel caliber estimation and can be considered a powerful biomarker of the vascular normalization induced by anti-angiogenic therapies (Emblem et al., 2013).

COMBINATION STRATEGIES

The partial effect of anti-angiogenic regimens in human cancers and the wide range of mechanisms sustaining intrinsic and acquired resistance represent a driving force for innovative strategies. For example, the anti-angiogenic regimens could improve their efficacy when associated with compounds targeting other major biological processes (e.g., tumor proliferation or apoptosis). In this context, the combination anti-angiogenic molecules with other approaches such as kinase inhibitors, chemotherapy, DNA repair inhibitors, radiotherapy, and immunotherapy have been reported in many experimental and human settings (Jayson et al., 2016).

Furthermore, nanotechnologies approaches could improve the current pharmacokinetic profiles of anti-angiogenic drugs and favor their selective accumulation in tumors and/or induce a shift the microenvironmental equilibria toward tumor-unfavorable conditions (El-Kenawi and El-Remessy, 2013).

Targeting Simultaneously VEGF and Other Angiogenic Mechanisms

Multiple inhibition of concomitant proangiogenic pathways may hamper cancer resistance or extend PFS. A first example deals the simultaneous or sequential blocking of the VEGF and Ang pathways in order to improve efficacy without increasing toxicity (Monk et al., 2014). The tyrosine kinase (TIE2) receptor is activated by its ligand Ang-1, which stabilizes vessels. Ang-2, which antagonizes Ang-1 effects, is highly expressed in cancer, destabilizing vessels and enabling sprouts under a chemotactic gradient of VEGFA. However, the scenario is more intricate

because Ang-2 has a partial agonist activity and has a pro-angiogenic effect independent of its cognate receptor TIE2. Increased amount of Ang-2 may be instrumental in eluding the anti-VEGF therapy. Preclinical and clinical studies in GMB reported that Ang-2 levels declined temporarily following inhibition of the VEGF pathway but later rebounded as tumors became resistant to the therapy (Batchelor et al., 2010; Chae et al., 2010). More recently it has been hypothesized that dual inhibition of VEGF and Ang-2 signaling respectively with TKI cediranib and MEDI3617 (an anti-Ang-2-neutralizing antibody) could prolong the temporal window of vascular normalization and thereby enhances the survival benefit of anti-VEGF therapy in two orthotopic murine model of GBM (Peterson et al., 2016). This combinatorial effect is related to an increased amount of recruited M1 polarized TAMs, which have anti-tumor effects. This observation is further supported by the data of another study showing that concurrent blockade of VEGF and Ang-2, using a bispecific Ang-2/VEGF antibody, similarly increased the M1/M2 ratio compared with VEGF-inhibition alone (Kloepper et al., 2016). These results match previous preclinical studies reporting a greater efficacy of combined VEGF and Ang-2 signaling inhibition as compared to single treatment (Brown et al., 2010; Hashizume et al., 2010; Koh et al., 2010; Daly et al., 2013; Kienast et al., 2013).

A second example is the association between VEGF blockade with therapies targeting FGF. Pan inhibitors of the FGF receptor (FGFR1-3), such as AZD4547 and BGJ398, elicited potent anti-tumor activities in preclinical investigations and are currently being evaluated in clinical trials (Chae et al., 2017). In this context, the dual inhibition of VEGFRs and FGFRs using brivanib produced enduring tumors stasis and angiogenic blockade following the failure of VEGF-targeted therapies (Allen et al., 2011).

A third approach exploits the possibility to target VEGF signals and Notch pathway, which is fundamental in regulation tip-stalk endothelial cell dynamics in sprouting angiogenesis (Jakobsson et al., 2009). Down-modulation of the Notch ligand Dll4 in combination with anti-VEGF therapy results in a greater tumor growth inhibition than with each agent alone in ovarian cancer models (Huang et al., 2016).

Fourth, HGF/c-MET pathway is driver and biomarker of VEGFR-inhibitor resistance in NSCLC. Dual VEGFR/c-MET pathway inhibition provide superior therapeutic benefit by delaying the onset of the resistant phenotype (Cascone et al., 2017). The efficacy of combining MET and VEGF inhibitors showed beneficial effect in murine GBM overexpressing MET (Okuda et al., 2017) and in pancreatic neuroendocrine tumors (Sennino et al., 2012).

Simultaneous inhibition of angiogenesis and vessel co-option may represent a further improvement of current therapeutic approaches. It has been recently reported that inhibition of angiogenesis and vessel co-option, by the knockdown of Arp2/3-mediated cancer cell motility, is more effective than targeting angiogenesis alone in a preclinical orthotopic model of advanced CRC liver metastasis (Frentzas et al., 2016).

Finally, tumor angiogenesis may be also affected and regulated by TGFβ family members, that exert a contradictory role in endothelial cells by inhibiting cell migration and proliferation but also acting as a proangiogenic factor and cooperating with VEGF, PDGF, and FGF in autocrine/paracrine signaling (Guerrero and McCarty, 2017). Preclinical studies have shown the anti-angiogenic effect elicited by the TGFβ inhibition in HCC, CRC, and GBM xenografts (Mazzocca et al., 2009; Zhang et al., 2011; Akbari et al., 2014) offering the rationale for the combination of TGFβ inhibitors with VEGF targeting agents (Neuzillet et al., 2015). In particular, are under clinical investigation the efficacy of the combination of galunisertib, a small molecule inhibitor of TGFβRI, with sorafenib and ramucirumab in HCC and PF-03446962, a monoclonal antibody against TGFβ, in combination with regorafenib in CRC.

Targeting Simultaneously VEGF and Oncogenic Drivers

Different oncogenic hits can perturb the balance between pro- an anti-angiogenic molecules thereby promoting pathological angiogenesis (Arbiser, 2004). For example, MAPK and PI3K-AKT pathways, which are often altered in cancers, are strictly connected with an increased transcription or translation of angiogenic factors. Consequently, specific inhibitors of signaling nodes of these pathways can induce vascular normalization and improve blood perfusion and tumor oxygenation (Qayum et al., 2009).

In particular, RAS activation increases VEGF and IL8 levels and the inhibition of RAS activity by gene silencing suppresses VEGF expression (Mizukami et al., 2005; Matsuo et al., 2009). Moreover, when VEGF expression is inhibited in CRC cells harboring KRAS mutations it has been reported a reduction of *in vivo* tumorigenic potential, highlighting the relevance of VEGF in exploiting the oncogenic potential of mutated KRAS (Okada et al., 1998). The role of KRAS in supporting angiogenesis is confirmed in NSCLC, where VEGF expression correlates with KRAS activating mutations (Konishi et al., 2000). We also described how mutated BRAF affected tumor angiogenesis and proved that targeting $BRAF^{V600E}$ stabilized the tumor vascular bed and abrogated hypoxia in mouse xenografts (Bottos et al., 2012). It has been suggested that EGFR-driven intracellular signaling may control angiogenesis and pharmacological inhibition of EGFR reduces VEGF expression in cancer cells (Ciardiello et al., 2001). It has been reported that a mechanism of acquired resistance to EGFR inhibitors is mediated by the increased secretion of VEGF, suggesting a key role for tumor-induced angiogenesis in the development of anti-EGFR resistance (Ciardiello et al., 2004). In NSCLC preclinical models it was found possible overcome acquired resistance to EGFR inhibitors by adding a VEGF blocker (Naumov et al., 2009). Human epidermal growth factor receptor 2 (HER2) is an oncogene overexpressed in more malignant breast cancer. Trastuzumab, which targets HER2-positive tumors strongly affect vascular shape and function and caused vessel normalization, down-regulating the secretion of VEGF and Ang-1 and in parallel up-regulating the expression of the anti-angiogenic factor thrombospondin 1 (Izumi et al., 2002).

These data suggest that pharmacological inhibition of oncogenes in tumor cells can restore a functional vasculature and potentially blocks the specific angiogenic program activated by individual tumors. Alternative strategy to target tumor angiogenesis could rescue the equilibrium of angiogenic signals by targeting the mutated oncogenes, which play a central role in this process. In order to potentially reduce acquired resistance combined strategy of anti-angiogenic and target therapies are explored in the recent years in pre-clinical and clinical trials.

Cetuximab and panitumumab are monoclonal antibodies that block the activation of EGFR and downstream RAS-RAF-MAPK and the PTEN-PIK3CA-AKT pathways (Ciardiello and Tortora, 2008; **Figure 2**). These two drugs are currently approved for the treatment of mCRC patients with all-*RAS* wild-type tumors. It has been recently reported that combined treatment with cetuximab and regorafenib induced synergistic anti-proliferative and pro-apoptotic effects by blocking MAPK and AKT pathways in orthotopic CRC xenograft models with primary or acquired resistance to anti-EGFR (Napolitano et al., 2015). This beneficial effect can be dependent on the inhibitor activities of regorafenib on different tyrosine kinase receptors involved in angiogenesis and potentially in the mechanism of resistance to cetuximab. The results provide the rationale for the clinical development of this combination. A phase I study was designed to evaluate the antitumor property of this combination among patients with advanced cancer refractory to several lines of therapy (**Table 2**). This study demonstrated that the combination of regorafenib and cetuximab showed a clinical benefit in all patients. It a plausible that inhibition of one of the molecular targets of regorafenib contributes to overcome resistance to previous anti-VEGF or anti-EGFR therapy (Subbiah et al., 2017). These results sustain the results of a previous work showing the cooperative antitumor activity of cetuximab or erlotinib and sorafenib in a xenograft model of NSCLC (Martinelli et al., 2010). More recently, it has been also shown the prolonged antitumor activity exerted by the combination of erlotinib with bevacizumab in a xenograft model of EGFR-mutated NSCLC (Masuda et al., 2017).

In the clinical setting, several studies are exploring the possibility of combining anti-EGFR drugs such as cetuximab, panitumumab or erlotinib, with different antiangiogenic drugs, including bevacizumab or sorafenib (**Table 2**). The results in unselected NSCLC or CRC cancer patients have been contradictory. Two large randomized phase III studies have evaluated the efficacy of the addition of cetuximab (CAIRO-2) or panitumumab (PACCE) to an oxaliplatin-containing chemotherapy doublet plus bevacizumab (Hecht et al., 2009; Tol et al., 2009). Both studies showed that the addition of the anti-EGFR antibodies did not improve the therapeutic efficacy. However, the results of randomized phase II study in NSCLC cancer patients selected for the presence of activating EGFR gene mutations demonstrated a clinically relevant increase of PFS by the combined treatment with erlotinib associated with bevacizumab compared erlotinib alone (Seto

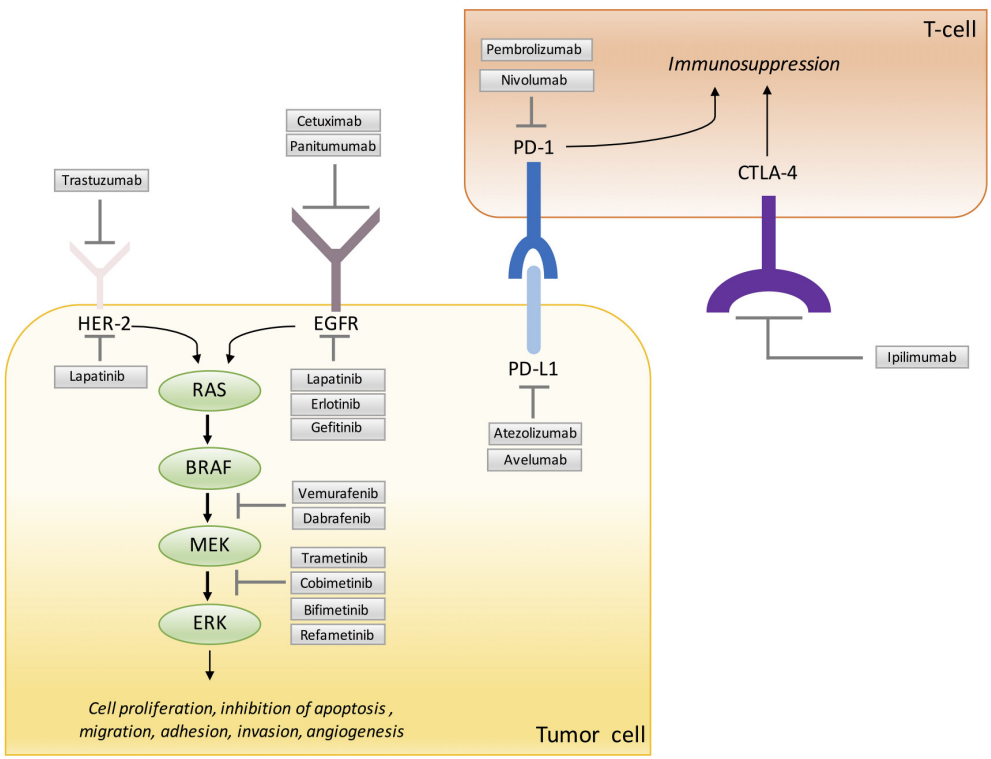

FIGURE 2 | Signaling molecules and immune checkpoint blocked by targeted therapy.

TABLE 2 | Selected Clinical Trials of VEGF-targeted therapy in combination with oncogene-targeted therapy (July 2017).

Anti-angiogenic	Target Therapy	Phase	Indications	ClinicalTrials.gov Identifier
Bevacizumab	Trastuzumab	2	Stage IV metastatic breast cancer	NCT00428922
Bevacizumab	Trastuzumab	3	Metastatic HER2+ breast cancer	NCT00391092
Bevacizumab	Trastuzumab	2	Breast cancer	NCT01321775
Bevacizumab	Trastuzumab	2	Metastatic HER2+ breast cancer	NCT00364611
Bevacizumab	Trastuzumab	2	Metastatic HER2+ breast cancer	NCT00670982
Bevacizumab	Trastuzumab	2	Metastatic HER2+ breast cancer	NCT00392392
Bevacizumab	Trastuzumab	2	Metastatic breast cancer	NCT00405938
Sorafenib	Trametinib	1	HCC	NCT02292173
Sorafenib	Refametinib	2	HCC	NCT01204177
Sorafenib	Refametinib	2	HCC RAS-mutated	NCT01915602
Regorafenib	Refametinib	1	Neoplasm	NCT02168777
Bevacizumab	Erlotinib	3	CRC	NCT00265824
Bevacizumab	Erlotinib	2	NSCLC EGFR-mutated	NCT01562028
Bevacizumab	Erlotinib	2	NSCLC EGFR-mutated	NCT01532089
Regorafenib	Cetuximab	1	Advanced cancers	NCT02095054
Sorafenib	Cetuximab	2	Squamos cell carcinoma of the Head and Neck	NCT00815295
Sorafenib	Cetuximab	2	CRC	NCT00326495
Bevacizumab	Trastuzumab	3	HER2-positive breast cancer	NCT00625898
Pazopanib	Lapatinib	2	HER2-positive breast cancer	NCT00558103.

CRC, colorectal cancer; EGFR, epiderma growth factor receptor; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor 2 ; NSCLC, non-small cell lung cancer. For reference see https://clinicaltrials.gov.

et al., 2014). This study provided the first evidence that the addition of bevacizumab to erlotinib confers a significant clinical improvement when used as first-line treatment for patients with NSCLC carrying activating EGFR mutations. More recently, the BELIEF trial (NCT01562028) provided further evidences of benefit for the combined use of erlotinib and bevacizumab in patients with EGFR-mutant NSCLC. Of notice, this study was stratified by the presence of the pretreatment of the T790M mutation with EGFR TKI (Rosell et al., 2017). Further, the efficacy and safety of sorafenib and cetuximab association are under evaluation also in patients with head and neck cancer and CRC (**Table 2**).

It has been demonstrated a positive correlation between elevated HER2 and VEGF levels and the poor outcome of breast cancer (Konecny et al., 2004). Trastuzumab, a monoclonal antibody against HER2, induces normalization and regression of the vasculature in HER2-overexpressing human breast cancer xenografts by lowering the amount of expressed proangiogenic molecules while up-regulating thrombospondin-1, which has anti-angiogenic activity (Izumi et al., 2002; **Figure 2**). Moreover, in a breast cancer xenograft model, VEGF was found to be elevated in the trastuzumab-resistant group, and sensitivity to trastuzumab was restored upon treatment with bevacizumab (Rugo, 2004). The small molecule inhibitor lapatinib, which inhibits EGFR and HER2, associated with regorafenib showed a greater anti-tumor activity than the compound alone in xenograft models of CRC associated with a relevant reduction of angiogenesis (Zhang et al., 2017). The result of this study has provided the rationale for using HER2 and VEGF inhibitors in clinical practice. Two large phase III trials evaluated the efficacy of bevacizumab and trastuzumab with chemotherapy in $HER2^{+}$ metastatic breast cancer (BETH and AVAREL) (**Table 2**). A modest improvement was seen in PFS, but the most intriguing finding was a more specific benefit from bevacizumab in the subgroup of patients with high levels of plasmatic VEGFA (Gianni et al., 2013). Moreover, the inhibition of VEGFRs and PDGFRs by pazopanib has been assessed in a phase II trial in combination with lapatinib in HER2-positive breast cancer. In this study the combination of lapatinib and pazopanib was associated with a numerically higher response rate without increase in PFS (Cristofanilli et al., 2013; **Table 2**).

We have recently demonstrated that targeting the vascular compartment with bevacizumab modulated the response to $BRAF^{V600E}$ inhibition in melanoma and CRC xenograft models. The final result is a synergistic antitumor effect and a delay of the appearance of the acquired resistance to BRAF inhibition. Of interest, we highlighted that this effect is the result of two biological processes: 1) the recruitment of TAMs polarized toward an M1-like phenotype and 2) the stroma remodeling characterized by the reduction of collagen deposition and the number of activated and tumor associated fibroblasts (Comunanza et al., 2017).

In the recent years, there has been great interest in developing clinically effective small-molecule inhibitors of the Ras-Raf-MEK-ERK1/2 pathway (Samatar and Poulikakos, 2014). Recently, (Bridgeman et al., 2016) provide preclinical evidence that combining TKI, such as sunitinib or pazopanib, with a MEK inhibitor (MEKI) is a rationale and efficacious treatment regimen for RCC, showing a more effective suppression of

tumor growth and tumor angiogenesis *in vivo*. Furthermore, it has also been reported the enhanced antitumor activity of a new combination regimen containing MEK inhibitor, binimetinib (MEK162), paclitaxel and bevacizumab in platinum-relapsing ovarian patient-derived xenografts (PDX) (Ricci et al., 2017). These results support the ongoing clinical development of MEK inhibitors and VEGF targeted combination therapy (**Table 2**). In particular, a phase II clinical trial (NCT01204177) investigating refametinib, a potent MEK1/2 inhibitor, in combination with sorafenib as a first-line treatment for subjects with advanced HCC showed that the combination of the two drugs appeared clinically active. Of notice, the majority of patients who responded to this regimen had mutant KRAS tumors (Lim et al., 2014). Further clinical trials are currently being conducted to explore this observation (**Table 2**).

Targeting VEGF in Combination with Immunotherapy

Immune checkpoint inhibition is exploiting in various tumors with promising results. The programmed death protein 1 (PD-1), its ligand the programmed death ligand 1 (PD-L1) and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) are negative regulators of T-cell immune function (**Figure 2**). Direct stimulation of the immune system with immune checkpoint inhibitors, such as antibody against PD1-1/PD-L1 and CTLA-4 has been reported in multiple cancers, resulting in several promising clinical trials (Mahoney et al., 2015; Callahan et al., 2016). Despite these exciting results, clinical responses are of limited duration (Sharma and Allison, 2017). A challenging aspect for the development of immune-therapies will be their inclusion in the current therapeutic strategies. Notably, tumor vasculature is an important co-regulator of the immune system and different anti-angiogenesis pathways interact with antitumor immunity through multiple mechanisms (Motz et al., 2014). Of great interest, VEGF was recognized as one of the critical molecule of immune suppression. VEGF reduces adhesion molecules expression on endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) impairing leukocyte-endothelial interactions and leukocyte entry in injured tissues. This process results in a dysfunctional tumor vasculature and hinders the immune T effector cell infiltration into the tumors (Ohm, 2003; Motz et al., 2014) and correlates with increased PD-1 expression on CD8 T cells (Voron et al., 2015). In addition to direct effects on T cells, VEGF suppresses dendritic cell differentiation and activity (Gabrilovich et al., 1998) and expands T regulatory cell (Terme et al., 2013) and myeloid-derived suppressor cells (Huang et al., 2007). In patients with CRC bevacizumab improved the antigen-presenting capacity of circulating dendritic cells (Osada et al., 2008), revealing an additional mechanism for bevacizumab on immune functions in the context of checkpoint blockade. Interestingly, it has been recently shown that high serum levels of VEGF were associated with decreased OS in advanced melanoma patients treated with ipilimumab, an anti-CTLA4 antibody (Yuan et al., 2014). In line with this, VEGF was decreased in patients with metastatic melanoma responding to sequential anti-CTLA4 and anti-PD-1 therapy but increased in non-responders (Chen et al., 2016) indicating a mechanism of therapeutic resistance and a potential target to therapy (Ott et al., 2015; Voron et al., 2015).

Besides to the effects on tumor vasculature, VEGF blockade may have a positive impact on the immune mechanisms leading to an anti-tumor response and preclinical studies support the possibility to exploit anti-angiogenesis inhibitors in association with molecule regulating innate and adaptive immunity. It has been reported in preclinical models of melanoma that blockade of the VEGF/VEGFR2 pathway increased the anti-tumor activity of adoptively transferred T-cells (Shrimali et al., 2010) and the combination of blocking VEGFR2 by the specific monoclonal antibody DC101 with a cancer vaccination showed a great anti-tumor effect by favoring $CD8^+$ T cell recruitment and reducing the number of regulatory T cells, which have tumor immune-suppressive function (Huang et al., 2012).

The positive effect on immune response obtained by halting VEGF pathway can be further increased by combining the block of Ang-2. A bispecific antibody, which bind both VEGFA and Ang-2 showed a better effect as compared to the single block, in many pre-clinical models and synergized with PD-1 blockade. Mechanistically, the antagonistic effect on these two angiogenic molecules favors the vascular normalization with a more efficient recruitment of $CD8^+$ T, which is concomitantly characterized by the up-regulation of PD-L1 on perivascular T cells (Schmittnaegel et al., 2017).

Further, the addition of anti-PD1 antibody to the CXCR4 inhibitor AMD3100 and sorafenib augments the antitumor immune responses mediated by $CD8^+$ T cells in an orthotopic murine models of HCC. The triple association showed a significant activity both on primary tumors and on the lung metastatic spreading (Chen et al., 2015).

More recently, a preclinical study provided evidences that anti-PD-1 or anti PD-L1 therapy sensitized and prolonged the efficacy of antiangiogenic therapy, and conversely, antiangiogenic therapy improved anti-PD-L1 treatment by supporting vascular changes, such as vessel normalization and high endothelial venules formation, that facilitate enhanced cytotoxic T cell infiltration and subsequent tumor cell destruction (Allen et al., 2017).

Based on these preclinical and translational data supporting synergy between angiogenesis inhibitors and checkpoint blockers, multiple trials of combinatorial therapies are under way and some have produced encouraging results. For example a phase I trial data of combination of bevacizumab and ipilimumab in patients with advanced melanoma showed disease control and increased CD8 T-cell tumor infiltration, resulting in durable patient response of more than 6 months (Hodi et al., 2014; Ott et al., 2015).

Other clinical trials are evaluating the combination between anti-angiogenic regimens and antibody targeting PD1 (nivolumab, pembrolizumab) and PDL1 (MPDL-3280A) (**Table 3**).

TABLE 3 | Selected Clinical Trials of VEGF-targeted therapy in combination with immune checkpoint inhibitors (July 2017).

Anti-angiogenic	Immunotherapy	Phase	Indications	ClinicalTrials.gov Identifier
Bevacizumab	Ipilimumab	2	Melanoma	NCT01950390
Bevacizumab	Ipilimumab	1	Melanoma	NCT00790010
Bevacizumab	Atezolizumab	2	CRC	NCT02982694
Bevacizumab	Atezolizumab	2	Melanoma brain metastases	NCT03175432
Bevacizumab	Atezolizumab	2	RCC	NCT02724878
Bevacizumab	Atezolizumab	3	RCC	NCT02420821
Bevacizumab	Nivolumab	2	Ovarian, Fallopian Tube Or Peritoneal Cancer	NCT02873962
Bevacizumab	Nivolumab	3	Glioblastoma	NCT02017717
Bevacizumab	Nivolumab	1	NSCLC	NCT01454102
Bevacizumab	Nivolumab	1	RCC	NCT02210117
Bevacizumab	Pembrolizumab	2	RCC	NCT02348008
Bevacizumab	Pembrolizumab	1/2	NSCLC	NCT02039674
Bevacizumab	Pembrolizumab	2	Glioblastoma	NCT02337491
Bevacizumab	Pembrolizumab	2	Melanoma/NSCLC brain metastases	NCT02681549
Aflibercept	Pembrolizumab	1	Solid tumors	NCT02298959
Sunitinib	Nivolumab	1	RCC	NCT01472081
Axitinib	Pembrolizumab	3	RCC	NCT02853331
Axitinib	Avelumab	3	RCC	NCT02684006
Cabozantinib	Nivolumab	3	RCC	NCT03141177

CRC, colorectal cancer; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma.
For reference see https://clinicaltrials.gov.

CONCLUSION

Preclinical findings show that single-drug antiangiogenic therapy delayed tumor growth but it was unable to determine tumor regression (Jayson et al., 2016) and in general, clinical efficacy of anti-angiogenic agents is lower than that observed in preclinical cancer models (Ebos and Kerbel, 2011) with significant adverse effects. The mechanisms that restrain the therapeutic efficacy of anti-angiogenic drugs in cancer are still poor comprehended. Moreover, an essential issue in the smart development of these compound is the identification of predictive biomarkers to find responder and non-responder patients. However biomarkers that are predictive of response to anti-angiogenic therapy in patients remain elusive (Jain et al., 2009; Vasudev and Reynolds, 2014; Jayson et al., 2016) and the patients' stratification on the basis of the drivers mutations and on feature of transcriptomic landscape including both gene coding and non-coding RNAs could really ameliorate the selection of responder patients.

Furthermore, biomarkers analysis and identification could represent the rationale for novel and combinatorial approaches, which could improve the clinical outcome exerted by angiogenesis inhibition. In particular oncogenes and immune response play a central role in the regulation of tumor angiogenesis and for this reason represent two attractive targets to develop combinatorial strategies. Many preclinical studies encourage the clinical exploitation of this approach.

AUTHOR CONTRIBUTIONS

VC contributed to the research, figure design, and writing of manuscript. FB contributed to the research, editing, and overall design of manuscript.

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ALK1Fc Suppresses the Human Prostate Cancer Growth in *in Vitro* and *in Vivo* Preclinical Models

Letizia Astrologo[1‡], Eugenio Zoni[1, 2‡], Sofia Karkampouna[1, 3‡], Peter C. Gray[4†], Irena Klima[1], Joël Grosjean[1], Marie J. Goumans[3], Lukas J. A. C. Hawinkels[3, 5], Gabri van der Pluijm[2], Martin Spahn[1], George N. Thalmann[1], Peter ten Dijke[3] and Marianna Kruithof-de Julio[1, 2, 3]*

[1] Department of Urology and Department for BioMedical Research, Urology Research Laboratory, University of Bern, Bern, Switzerland, [2] Department of Urology, Leiden University Medical Centre, Leiden, Netherlands, [3] Department of Molecular Cell Biology, Cancer Genomics Center, Leiden University Medical Centre, Leiden, Netherlands, [4] Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, CA, United States, [5] Department of Gastroenterology-Hepatology, Leiden University Medical Centre, Leiden, Netherlands

Prostate cancer is the second most common cancer in men and lethality is normally associated with the consequences of metastasis rather than the primary tumor. Therefore, targeting the molecular pathways that underlie dissemination of primary tumor cells and the formation of metastases has a great clinical value. Bone morphogenetic proteins (BMPs) play a critical role in tumor progression and this study focuses on the role of BMP9- *A*ctivin receptor-*L*ike *K*inase 1 and 2 (ALK1 and ALK2) axis in prostate cancer. In order to study the effect of BMP9 *in vitro* and *in vivo* on cancer cells and tumor growth, we used a soluble chimeric protein consisting of the ALK1 extracellular domain (ECD) fused to human Fc (ALK1Fc) that prevents binding of BMP9 to its cell surface receptors and thereby blocks its ability to activate downstream signaling. ALK1Fc sequesters BMP9 and the closely related BMP10 while preserving the activation of ALK1 and ALK2 through other ligands. We show that ALK1Fc acts *in vitro* to decrease BMP9-mediated signaling and proliferation of prostate cancer cells with tumor initiating and metastatic potential. In line with these observations, we demonstrate that ALK1Fc also reduces tumor cell proliferation and tumor growth *in vivo* in an orthotopic transplantation model, as well as in the human patient derived xenograft BM18. Furthermore, we also provide evidence for crosstalk between BMP9 and NOTCH and find that ALK1Fc inhibits NOTCH signaling in human prostate cancer cells and blocks the induction of the NOTCH target Aldehyde dehydrogenase member ALDH1A1, which is a clinically relevant marker associated with poor survival and advanced-stage prostate cancer. Our study provides the first demonstration that ALK1Fc inhibits prostate cancer progression, identifying BMP9 as a putative therapeutic target and ALK1Fc as a potential therapy. Altogether, these findings support the validity of ongoing clinical development of drugs blocking ALK1 and ALK2 receptor activity.

Keywords: BMP9, ALK1, ALK2, ALK1Fc, NOTCH, prostate cancer

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****Correspondence:***
Marianna Kruithof-de Julio
marianna.kruithofdejulio@dbmr.unibe.ch

†Present Address:
Peter C. Gray, ScienceMedia Inc., San Diego, CA, United States

‡These authors have contributed equally to this work.

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INTRODUCTION

Prostate cancer is the second most common cancer in men worldwide (Jemal et al., 2010). Currently prostate cancer, when still in its first phase of androgen dependency, can be successfully treated surgically. However, if the tumor becomes androgen independent, therapy is no longer possible and lethality is almost invariably due to the consequences of metastasis. Therefore, understanding the molecular pathways that underlie the emergence and spread of metastases from primary tumors is of great biological and clinical value.

Expression of several BMPs has been examined in prostatic tissue with benign prostatic hyperplasia (BPH), non-metastatic and metastatic prostatic adenocarcinoma and has been associated with cancer aggressiveness (Ye et al., 2007; Ye and Jiang, 2016). Among the BMPs, BMP9 is one of the most recently identified (Song et al., 1995). Little is known about the roles of BMP9 and its cell surface signaling receptors, ALK1 and ALK2, in prostate cancer and particularly in androgen independent and metastatic prostate cancer. Current research has not only attributed a tumor-promoting role to BMP9 (Herrera et al., 2009, 2013; Li et al., 2014) but also tumor suppressing properties (Ye et al., 2008; Wang et al., 2011; Olsen et al., 2014) in different types of cancer, including prostate cancer.

Previous studies have highlighted the role of ALK1, which is predominantly expressed by endothelial cells (van Meeteren et al., 2012), as key regulator of angiogenesis in normal tissue and in tumors (Hawinkels et al., 2013; Bendell et al., 2014). BMP9 and BMP10 are high affinity ligands for ALK1, while BMP9 signals through the BMP type I receptor ALK2 (David et al., 2007; Herrera et al., 2009; Bragdon et al., 2011). Binding of BMP9/BMP10 to ALK1/ALK2 results in phosphorylation and activation of downstream effectors SMAD1 and/or SMAD5 (David et al., 2007; Scharpfenecker et al., 2007; Herrera et al., 2009). BMP9 promotes human epithelial ovarian cancer and human immortalized ovarian surface epithelial cell proliferation through ALK2/SMAD1/SMAD4 pathway (Herrera et al., 2009). Similarly, BMP9 stimulates proliferation of liver cancer cells (Herrera et al., 2013) and osteosarcoma growth (Li et al., 2014).

Several studies have highlighted the role of BMP9/ALK1 in blood vessel formation, outlining its critical involvement in pathological and tumor angiogenesis (Urness et al., 2000; Cunha and Pietras, 2011). Interestingly, alterations of signal transduction pathways that are important for blood vessel formation, such as the NOTCH pathway, have also been associated with arterio-venous malformations (Gale et al., 2004; Krebs et al., 2004). Recently, BMP9 and BMP10 signaling were linked to NOTCH signaling, one of the major pathways involved in prostate cancer development, progression and bone metastasis (Carvalho et al., 2014; Kron et al., 2017; Zhang et al., 2017). Expression profiling studies have shown that members of the NOTCH pathway are characteristic of high-grade (Gleason $4 + 4 = 8$) micro-dissected prostate cancer cells compared to low-grade (Gleason $3 + 3 = 6$) (Ross et al., 2011). Moreover, inhibition of NOTCH1 reduces prostate cancer cell growth, migration and invasion (Wang et al., 2010). Interestingly, the NOTCH signaling indirectly activates the enzymatic activity of ALDH1A1, a well-known marker of prostate cancer stem cells (Ginestier et al., 2007; Li et al., 2010; Le Magnen et al., 2013; Zhao et al., 2014), which are thought to be responsible for tumor recurrence, metastasis and cancer related death (Moltzahn and Thalmann, 2013).

In order to understand the role of BMP9 in prostate cancer progression, we employed the soluble chimeric protein ALK1Fc (ACE-041) (Seehra et al., 2009) which binds BMP9 and BMP10 with high affinity and blocks their signaling via ALK1 and ALK2 receptors by acting as a ligand trap (Cunha et al., 2010; Mitchell et al., 2010). Phase I clinical trials have been completed using ALK1Fc as anti-angiogenesis therapy in myeloma (clinicaltrials.gov identifier NCT00996957). Here we show that ALK1Fc reduces BMP9 signaling and decreases proliferation of highly metastatic human prostate cancer cells *in vitro*. We further demonstrate that ALK1Fc impairs tumor angiogenesis, affects tumor cell proliferation and reduces tumor growth *in vivo*. Taken together these data suggest BMP9 as a possible therapeutic target in prostate cancer and provide a new rationale for ongoing clinical development of drugs blocking BMP9 signaling via ALK1 and ALK2.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The human osteotropic prostate cancer cell line PC-3M-Pro4-Luc2 (Kroon et al., 2014; Zoni et al., 2015, 2017) was maintained in DMEM supplemented with 10% FCII, 0.8 mg/ml Neomycin (Santacruz, Dallas, USA) and 1% Penicillin-Streptomycin (Life Technologies, Carlsbad, USA).

Recombinant Proteins and Chemical Inhibitors

ALK1Fc (de Vinuesa et al., 2016) is a fusion protein comprised of the extracellular domain (ECD) of human ALK1 fused to the Fc region of IgG and was obtained from Acceleron Pharma, Cambridge, USA. As a control we used either the Fc domain of IgG_1 (MOPC-21; Bio Express, West Lebanon NH) or normal goat IgG from R&D System.

Recombinant human BMP9 was obtained from R&D System, whereas the chemical inhibitor LDN193189 was purchased from Axon Medchem. The final concentration for the *in vitro* experiments was 1 nM for BMP9 and 120 nM for LDN193189.

Lentiviral-Mediated RNA Interference of NOTCH1

shRNAi for NOTCH1 (TRC000000350253, TRC000000350330, TRC0000003361, TRC0000003360) were obtained from Sigma MISSION library and used for lentiviral vector production and transduction as described previously (Zoni et al., 2017). Scramble shRNA (NT; SHC002, Sigma) was used as control. The transduced cells underwent puromycin selection and used for further experiments as described below. The experiments were carried out in accordance with standard biosecurity procedures.

Luciferase Reporter Assays and Constructs

PC-3MPro4 cells were seeded at density of 50,000 cells in 500 μL medium in a 24-well plate. Transient transfection of reporter constructs was performed with Lipofectamine2000 (Life Technologies) according to the manufacturer's protocol. For each well, 100 ng of NICD-ff-luciferase, 10 ng CAGGS-Renilla luciferase, 100 ng BRE renilla (Korchynskyi and ten Dijke, 2002) and 100 ng BRELuc/well were transfected. After 24 h, medium was replaced and cells were treated with BMP9 for 24 h. The *Firefly* luciferase and *Renilla* luciferase levels in the lysates were measured using Dual Luciferase Assay (Promega, Madison, USA).

RNA Isolation and Real-Time Q-PCR

Total RNA was isolated from PC-3M-Pro4-Luc2 cells with Trizol Reagent (Invitrogen, Waltham, USA) and cDNA was synthesized by reverse transcription (Promega, Madison, USA) according to the manufacturer's protocol. qRT-PCR was performed with Biorad CFX96 system (Biorad, Veenendaal, The Netherlands). Gene expression was normalized to *GAPDH* or *β-actin*. Total RNA from frozen section (5 μm) was isolated with Qiagen Mini Isolation kit (Venlo, The Netherlands) according to the manufacturer's protocol. Primer sequences are listed in Supplementary Table I.

MTS Assay

Cells were seeded at density of 2,000 cells/well in low serum condition (0.3% FCII), treated with ALK1Fc or Control-Fc (CFc) (10 μg/ml, Acceleron, USA) and allowed to grow for 24, 48, 72, and 96 h. After incubation, 20 μl of 3-(4,5 dimethylthiazol- 2-yl)- 5 -(3 -carboxymethoxyphenyl)- 2 -(4 -sulfophenyl)- 2 H-tetrazolium (MTS) was added and mitochondrial activity was measured after 2 h incubation at 37°C. MTS absorbance values are positively proportional to total number of metabolically active cells providing an indirect correlation with cell proliferation rate (CellTiter96 Aqueous Non-radioactive Cell proliferation assay, Promega) (Berridge et al., 2005).

Animals

Male 6–8 week-old athymic nude (Balb/c *nu/nu*) or CB17 SCID mice, purchased from Charles River (L'Arbresle, France), were used in all *in vivo* experiments. Mice were housed in individual ventilated cages under sterile condition, and sterile food and water were provided *ad libitum*. Animal experiments were approved by the local committee for animal health ethics and research of Leiden University (DEC #11246) and Canton of Bern, Switzerland (Permit Number: BE55/16), and carried out in accordance with European Communities Council Directive 86/609/EEC and Swiss Guidelines for the Care and Use of Laboratory Animals.

Orthotopic Prostate Transplantation and ALK1Fc Treatment

25,000 PC-3M-Pro4-Luc2 cells (10 μl final volume) were injected in the dorsal prostate lobe of anesthetized male nude mice. In brief: After anesthetizing the mice with isoflurane, each mouse was placed on its back and a small incision was made along the lower midline of the peritoneum for about 1 cm. The prostate dorsal lobes were exteriorized and stabilized gently. A 30-gauge needle attached to a 1-cc syringe was inserted into the right dorsal lobe of the prostate. 10 μl of the cell suspension was slowly injected. A well-localized bleb indicates a successful injection. After retracting the needle, a Q-tip was placed over the injection site for about 1 min to prevent bleeding and spillage of material. The prostate was then returned to the peritoneum and the abdominal wall and skin layer was sutured. After establishment of the primary tumor, at 10 days after the orthotopic transplantation, mice were intraperitoneally injected with Control-Fc (CFc) or ALK1Fc compounds (10 mg/kg) twice per week. Administration of compounds was performed for 4 weeks. After the experimental periods, mice were injected with hypoxia probe (6 mg/kg, Burlington, Massachusetts, USA) and lectin-Tomato (1 mg/kg, Vector Laboratories, USA) intravenously prior to perfusion and sacrificed according to our mouse protocol. Tumors were dissected and processed for further histomorphological analysis as described below.

Subcutaneously BM18 Transplantation and ALK1Fc Treatment

BM18 xenografts were transplanted subcutaneously in CB17 SCID mice anesthetized with a cocktail of medetomidin (1 mg/kg body weight), midazolam (10 mg/kg) and fentanyl (0.1 mg/kg) (Schwaninger et al., 2007). After 1 week, the animals were intraperitoneally injected with ALK1Fc or IgG at the dose of 10 mg/kg once a week, for 5 weeks. Every week the tumors were measured with the caliper and finally dissected and fixed in 4% paraformaldehyde (PFA) for paraffin embedding and hematoxylin and eosin staining.

Whole Body Bioluminescent Imaging (BLI)

Tumor growth from orthotopic injection was monitored weekly by whole body bioluminescent imaging (BLI) using an intensified-charge-coupled device (I-CCD) video camera of the *in vivo* Imaging System (IVIS100, Xenogen/Perkin Elmer, Alameda, CA, USA) as described previously (Buijs et al., 2007; van den Hoogen et al., 2010). In the orthotopic transplantation experiment the newer IVIS Lumina II (Xenogen/Perkin Elmer, Alameda, CA, USA) was used for BLI measurements. Mice were anesthetized using isoflurane and injected intraperitoneally with 2 mg D-luciferin (Per bio Science Nederland B.V., Etten-Leur, the Netherlands). Analyses for each metastatic site were performed after definition of the region of interest and quantified with Living Image 4.2 (Caliper Life Sciences, Teralfene, Belgium). Values are expressed as relative light units (RLU) in photons/s.

Immunofluorescence

Immunofluorescence staining was performed on 5-μm paraffin embedded sections. For antigen retrieval, sections were boiled in antigen unmasking solution (Vector Labs, Peterborough, UK) and stained with anti- pH3 (Millipore), cleaved CASP3 (Cell Signaling), CD31 (Sigma) or ALDH1A1 (Abcam) antibodies. Sections were blocked with 1% bovine serum albumin (BSA)-PBS-0.1% v/v Tween-20 and incubated with primary antibodies diluted in the blocking solution, overnight at 4°C. Sections were

then incubated with secondary antibodies labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes, Waltham, USA) at 1:250 in PBS-0.1% Tween-20. Nuclei were visualized by TO-PRO3 (Invitrogen/Molecular Probes, 1:1000 diluted in PBS-0.1% Tween-20) (Karkampouna et al., 2014).

Western Immunoblotting

Cell lysates were prepared using RIPA buffer (Thermo Scientific) and protein concentrations were quantified according to manufacturer's protocol (Thermo Scientific). Proteins (20 μg per sample) were separated by 15% SDS-PAGE followed by transfer to a blotting membrane. The membrane was blocked with 5% Milk, dissolved in PBS-Tween, for 1 h at room temperature. The membrane was incubated with 1:1,000 primary antibody (anti-NOTCH1, Cell Signaling, catalog number 3608) at 4°C overnight. Subsequently, the membrane was incubated with 1:10,000 secondary horseradish peroxidase (HRP) antibody. All antibodies were dissolved in PBS-Tween. Chemiluminescence was used to visualize the bands.

Clonogenic Assay

Clonogenic assay was performed in 6 well plate. 100 cells were seeded in 2 mL of medium and incubated at 37°C in presence of 5% CO_2 for 2 weeks. Plates were washed with PBS and cells fixed for 5 min with a solution of 4% PFA. Colonies were stained with 0.1% crystal violet (Sigma-Aldrich, The Netherlands) and plates were imaged before processing the data with ImageJ software (Franken et al., 2006; Rafehi et al., 2011; Guzman et al., 2014).

Prostate Cancer Dataset Analysis

The Taylor MSKCC prostate dataset was queried for BMP9 and ALK1 expression in prostate cancer patients through the online biomarker validation tool SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp). The data are censored as "recurrence month," and the risk groups are defined estimating a prognostic index by the Cox model algorithm (Aguirre-Gamboa et al., 2013).

R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used to investigate the ALK2, JAG1 and NOTCH1 expression in benign ($n = 48$) vs. tumor ($n = 47$) tissues using the GEO accession number GSE29079 dataset (Borno et al., 2012).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad software) using *t*-test or ANOVA for comparison between more groups. Data is presented as mean ± SEM. *P*-values < 0.05 were considered to be statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

High BMP9 and ALK1 Correlate with Recurrence in Prostate Cancer Patients

The role of BMP9 in cancer development and progression is still controversial. We analyzed a Taylor MSKCC Prostate dataset (GSE21032) through the online tool SurvExpress to assess how the expression of BMP9 and ALK1 is related with recurrence in prostate cancer. We found that the group of patients with high expression of BMP9 and ALK1 has higher probability to encounter biochemical recurrence than the group with lower levels of BMP9 and ALK1 (**Figures 1A,B** top). The SurvExpress tool also defines "high-" and "low-" risk group of patients based on the risk prognosis calculated as described in the Material and Methods. According to that definition, we also found that the high-risk group of patients had higher expression of BMP9 and ALK1 than lower risk patients (**Figures 1A,B** bottom). Therefore, we explored whether inhibition of BMP9 signaling in mouse models of prostate cancer interferes with tumor growth.

ALK1Fc Reduces Primary Prostate Tumor Burden *in Vivo*

To investigate the role of BMP9 in prostate cancer progression, the BMP9 ligand trap ALK1Fc was administered in an orthotopic mouse model of prostate cancer. Primary prostate tumor growth was induced by intra-prostatic inoculation of human prostate cancer PC-3M-Pro4-Luc2 cells in Balb/c nude mice and tumor progression was followed by bioluminescence imaging (BLI) (Kroon et al., 2014) (**Figure 2A**). Based on the BLI signal the mice were randomized in two treatment groups: ALK1Fc or control (C) Fc ($n = 15$ per group). The recombinant proteins were injected twice weekly and tumor imaging and body weights were monitored weekly for 5 weeks (Supplementary Figure 1). Tumor burden was quantitatively assessed for each animal during the course of treatment. The group of animals that received ALK1Fc exhibited smaller tumor size compared to the animals that received CFc based on bioluminescence quantification (**Figure 2B**, $p < 0.01$).

ALK1Fc Reduces Cell Proliferation in the Primary Prostate Tumor

The degree of tumor angiogenesis is critical for progressive tumor growth beyond a few mm^3 in size. Intravital lectin perfusion was used to map the perfused elements of the tumor vasculature in mice. Fluorescent-conjugated lectin (lectin-Tomato) was visualized in tumor tissue sections and quantified. We observed a trend in decreased vascular density, indicated by the overall lectin presence, in the tumors treated with ALK1Fc compared to the CFc group (Supplementary Figures 2A,B). We evaluated the presence of endothelial cells in tumor sections by CD31 immunofluorescence. A trend of decreased CD31 expression was also observed after treatment with ALK1Fc suggesting fewer endothelial cells and vessels (Supplementary Figures 2C,D). Hypoxia is an important component of angiogenesis and critical for tumor formation. A hypoxia-induced probe was injected in tumor bearing mice just prior to sacrifice and the hypoxic areas within the tumors were visualized after tumor resection (**Figure 3A**; left panels). Although hypoxic areas were found in both treatment groups, the overall amount of hypoxia seemed to be higher in ALK1Fc-treated mice relative to the CFc-treated mice (**Figure 3A**; right graph, $p = 0.050$). We assessed the presence of cell proliferation and cell death in these tumors by immunofluorescence for the mitosis marker phosphorylated histone 3 (PH3) and the apoptosis marker

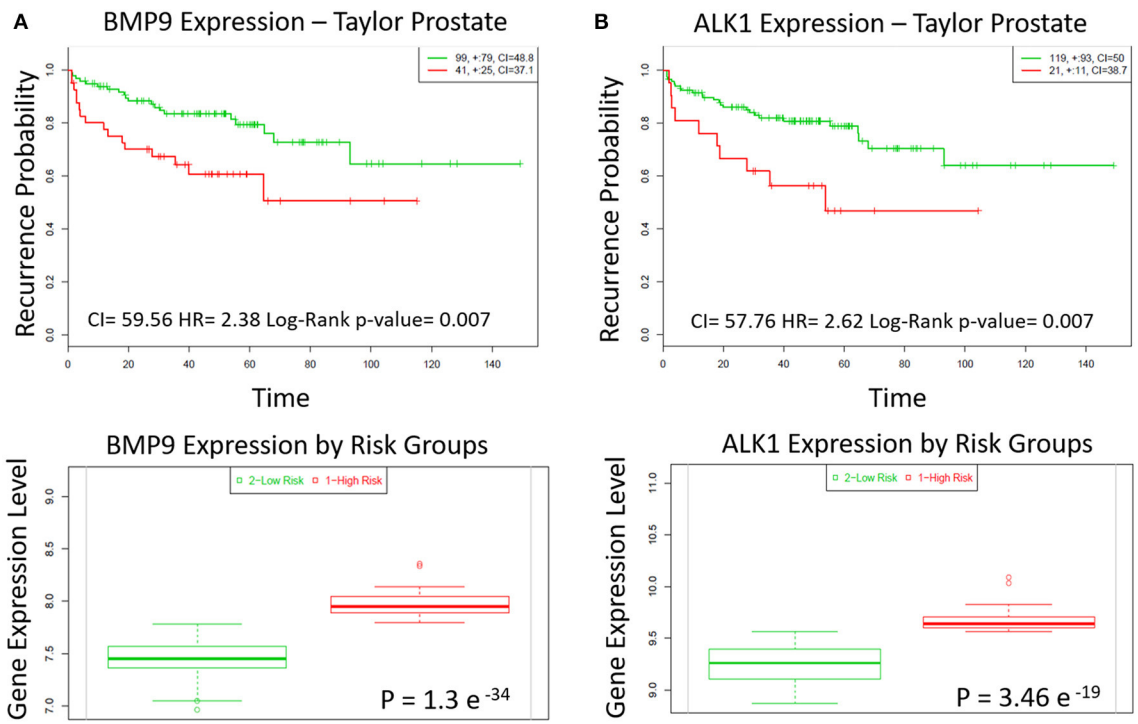

FIGURE 1 | BMP9 and ALK1 correlate with poor patient prognosis. **(A,B)** Top panels: Kaplan-Meier survival curves of censored Cox analysis in Taylor-MSKCC prostate database stratified by maximized BMP9 and ALK1 expression risk groups. Subjects with a higher expression display earlier biochemical recurrence than subjects with a lower risk (Bovelstad and Borgan, 2011). Red, high expression; Green, low expression. CI, Concordance Index; HR, Risk Group Hazard Ratio. Bottom panels: BMP9 and ALK1 expression levels stratified by risk groups. Red, high Risk; Green, low risk.

cleaved caspase 3 (CASP3), respectively. Dividing PH3 positive cells are predominantly located in normoxic areas (**Figure 3A**; left panel). Quantification of immunofluorescence signal shows that the number of dividing cells is lower in the ALK1Fc-treated animals (**Figure 3A**; right graph $p < 0.05$). Detection of apoptotic cells (Caspase-3 positive) is higher in the ALK1Fc-treated tumors (**Figure 3A**; right graph $p < 0.05$) and occurs mostly, but not exclusively, in hypoxic areas (**Figure 3A**; left panel), suggesting a correlation between the hypoxia and tumor cell death.

ALK1Fc Decreases Proliferation of Human Prostate Cancer Cells *in Vitro*

To investigate how ALK1Fc can decrease tumor growth, we studied the effect of ALK1Fc on prostate cancer cells. We measured the mRNA levels of the BMP9 type I receptors ALK1 and ALK2 in the PC-3M-Pro4-Luc2 (Kroon et al., 2014) human prostate cancer cell line and tested their response to BMP9. Consistent with a previous report in highly metastatic PC-3 and PC-3M prostate cancer cells (Craft et al., 2007), qRT-PCR analysis in osteotropic PC-3M-Pro4-Luc2 cells revealed undetectable levels of ALK1 but measurable levels of ALK2 (Supplementary Figure 3A). Treatment with BMP9 showed a dose-dependent induction of BRE-*Renilla* luciferase (luc) activity in PC-3M-Pro4 cells (p-value = 0.005 and 0.05 with 0.5 nM and 1 nM BMP9, respectively) indicative of conserved and active canonical Smad signaling machinery (Supplementary Figure 3B). We subsequently tested the combined effect of treating cells with 1 nM BMP9 and either ALK1Fc or CFc on BRE reporter assay and found that treatment with ALK1Fc (10 μg/mL) completely abolished BMP9 signaling (Supplementary Figure 3C**;** BMP9+ALK1Fc) as evidenced by BRE-luc activity levels similar to that of cells without BMP9 treatment (Untreated). Treatment with BMP9+CFc (10 μg/mL) led to induction of BRE-luc activity that was similar to the level of BMP9 treatment alone (Supplementary Figure 3C**;** p-value < 0.05). Taken together, these results indicate that ALK1Fc blocks BMP9 signaling via ALK2 in PC-3MPro4 cells.

Moreover, ALK1Fc treatment, but not CFc, strongly reduced BMP9-induced cell proliferation in PC-3M-Pro4-Luc2 ($p < 0.001$ at day 4 comparing vehicle vs. BMP9 or ALK1Fc treatment, respectively; **Figure 3B**). This effect appeared to be specific (Supplementary Figure 3D) since treatment of PC-3M-Pro4-Luc2 cells with BMP9 in combination with an ALK2 small molecule kinase inhibitor (LDN193189, LDN) (Cuny et al., 2008; Shi et al., 2011) similarly resulted in the complete loss of BMP9-induced cell proliferation (**Figure 3C**). LDN treatment also blocked BMP9 stimulation of the BRE-luc reporter in PC-3M-Pro4-Luc2 cells (Supplementary Figure 3E). Together, these data indicate that ALK1Fc strongly reduces BMP9- induced proliferation in human prostate cancer cells.

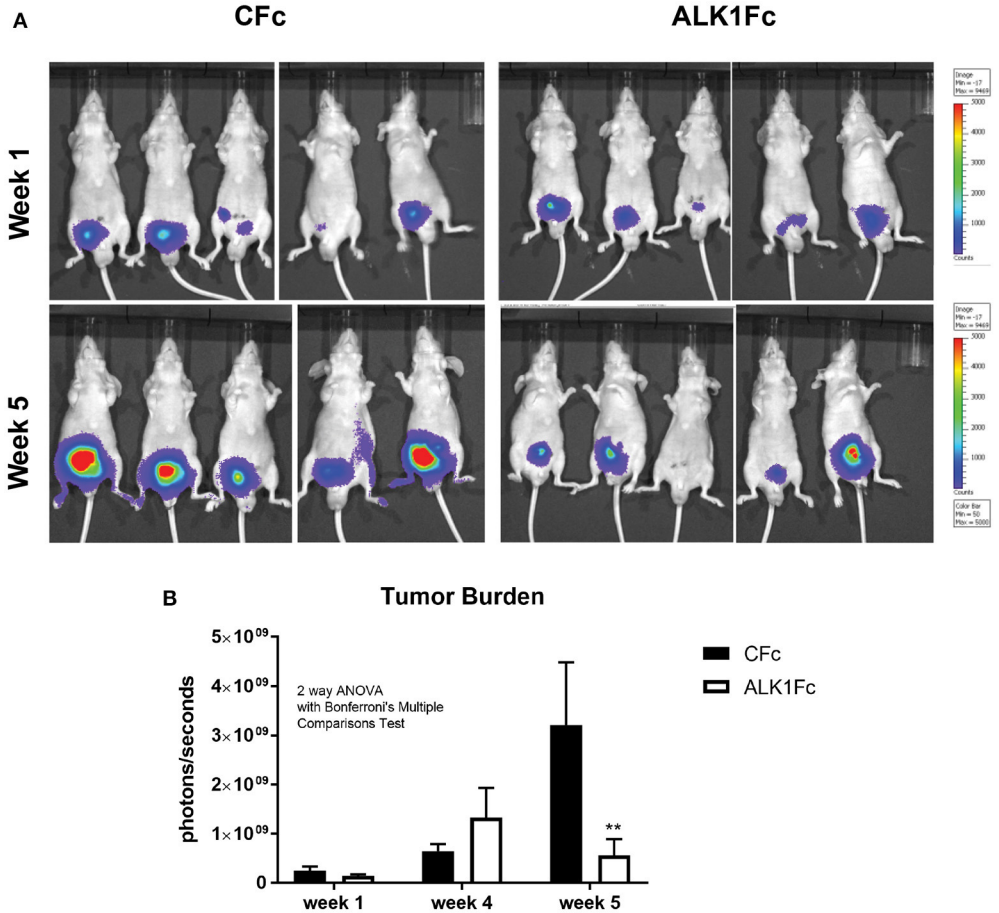

FIGURE 2 | ALK1FC reduces primary prostate tumor burden. **(A)** PC-3M-Pro4-Luc2 cells were orthotopically injected in the dorsal lobe of prostate glands of nude mice ($n = 15$ per group). Detection of primary tumor burden was observed at 2 weeks after injection, with the time point designated as "week 1" at the start of treatment with ALK1Fc or CFc. Representative examples of bioluminescent images of tumor burden at the start of treatment with ALK1Fc/CFc (week 1) and at the end point (week 5). **(B)** Quantification of bioluminescent signal (photons/sec) in mice treated with either CFc ($n = 14$) or ALK1Fc ($n = 15$) for 5 weeks. Error bars indicate ± SEM. $^{**}P$-value < 0.01.

ALK1Fc Inhibits ALDH1A1 Expression *in Vivo* and Interferes with NOTCH Signaling

Given its ability to reduce primary tumor burden and block BMP9-induced tumor cell proliferation *in vitro* and *in vivo*, we assessed the effects of ALK1Fc on the relative expression of *ALDH1A1*, a marker previously associated with cancer stem cell-like properties and poor patient prognosis (Li et al., 2010; Le Magnen et al., 2013). Treatment of prostate tumor bearing mice with ALK1Fc affected the number of ALDH1A1 positive cells in the prostate tumor tissues both at the protein (**Figure 4A**) and mRNA levels (**Figure 4B**). *In vitro* stimulation with BMP9 of the same cell line used to induce tumors in the xenograft mouse model confirmed that treatment with BMP9 or BMP9+CFc upregulates ALDH1A1 expression while BMP9+ALK1Fc treatment does not have any effect (**Figure 4C**).

We tested the effects of BMP9 treatment on the colony forming capacity of PC-3M-Pro4-Luc2 cells (**Figure 4D**) and found that it alters cell proliferation and strongly increasing the size of the colonies (**Figure 4E,** $p < 0.05$). However, BMP9 showed no effect on colony formation ability of PC-3M-Pro4-Luc2 since the total number of colonies formed with or without BMP9 treatment is similar (**Figure 4F**).

ALDH1A1 is known to be regulated by NOTCH signaling (Ginestier et al., 2007; Le Magnen et al., 2013; Zhao et al., 2014) and NOTCH1 plays a prominent role in prostate cancer cell proliferation and migration (Shou et al., 2001; Zhang et al., 2006; Leong and Gao, 2008; Bin Hafeez et al., 2009; Wang et al., 2010). Larrivee et al. have shown that ALK1 and NOTCH converge on common downstream pathways and that BMP9 treatment is sufficient to upregulate expression of the NOTCH pathway ligand JAG1 in HUVEC non-transformed cells (Larrivee et al., 2012).

To assess the clinical relevance of crosstalk between BMP9/ALK2 signaling and NOTCH pathway activation in human prostate cancer, we performed bioinformatics analysis in 48 benign prostate tumors and 47 malignant prostate tumors (Borno et al., 2012) using R2 data mining platform (source: GEO

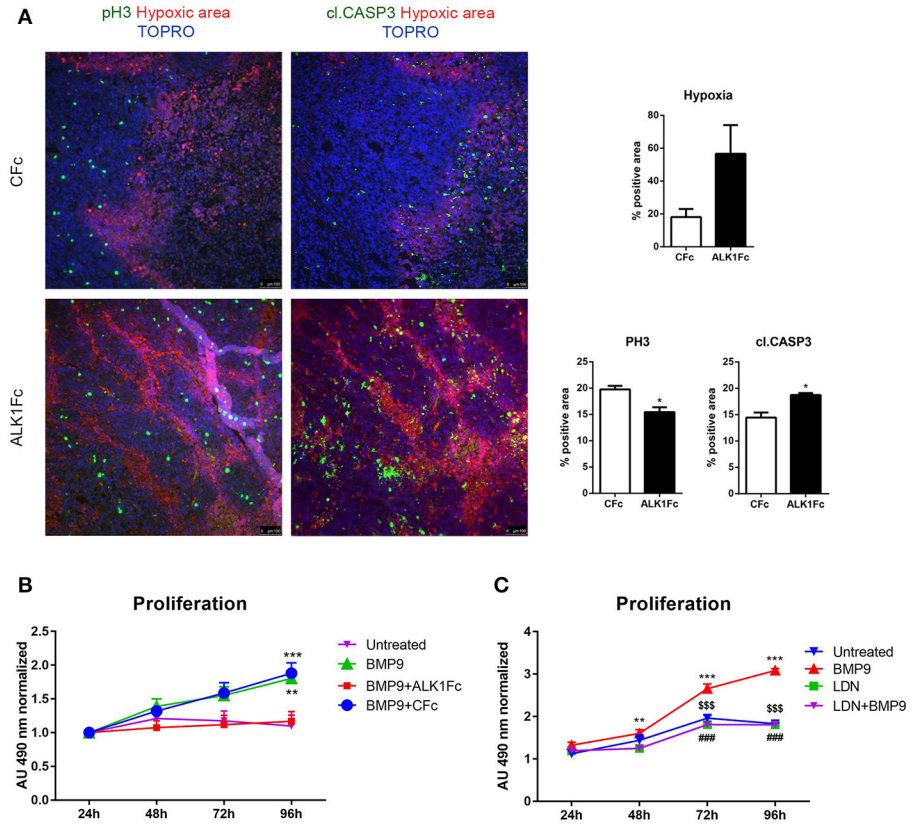

FIGURE 3 | ALK1Fc increases hypoxia and apoptosis and decreases cell proliferation *in vivo*. **(A)** Left panels: Representative images of hypoxia immunofluorescence staining (red) in primary prostate tumor samples after 5 weeks of treatment with either ALK1Fc or CFc. Hypoxia probe was injected prior to sacrifice and was detected by a specific fluorescent antibody. Immunofluorescence images for colocalization of apoptotic or proliferating cells in hypoxic/normoxic area within the prostate tumor area in ALK1Fc and CFc treated animals. pH3: PhosphoHistone 3 proliferation marker (green); cleaved caspase 3 apoptosis marker (green); Hypoxic probe-antibody: hypoxic area (red); TOPRO: nuclear dye (blue). Right graphs: Quantification of hypoxia, pH3 and cl.CASP3 positive area in all tumor samples of each group ($n = 6$ for CFc, $n = 7$ for ALK1Fc). **(B)** MTS assay (24, 48, 72, 96 h) was performed in PC-3M-Pro4-Luc2 cells stimulated with recombinant BMP9 (1 nM), BMP9 (1 nM) + ALK1Fc (10 μg/ml), or BMP9 (1 nM) + CFc (10 μg/ml). Accumulation of MTS was measured based on absorbance at 490 nm. Values are normalized to the basal measurements at 24 h after cell seeding and treatments. Graph represents values for three independent experiments ($n = 3$). Error bars indicate ± SEM. $^{**}P$-value < 0.01 BMP9 vs. Untreated and $^{***}P$-value < 0.001 BMP9+CFc vs. Untreated. **(C)** MTS assay (24, 48, 72, and 96 h) performed in PC-3M-Pro4-Luc2 cells seeded at low density in 96-well plates and treated with BMP9 (1 nM), LDN (BMP type I receptor inhibitor LDN193189, 120 nM) or LDN+BMP9. ($n = 2$). Values are normalized to the basal measurements at the time of cell seeding and treatments. Error bars indicate SEM. $^{**}P$-value < 0.01 BMP9 vs. LDN and BMP9 vs. LDN+-BMP9; $^{***}P$-value < 0.001 BMP9 vs. Untreated; $^{\$\$\$}$BMP9 vs. LDN; $^{\#\#\#}$BMP9 vs. LDN+BMP9; $^{*}P$-value < 0.05.

ID: GSE29079). Transcript levels of *ALK2*, the NOTCH ligand *JAG1*, and *NOTCH1* were significantly higher in the malignant tumor group compared to the benign group (**Figures 5A–C**).

We targeted the expression of NOTCH1 in PC-3M-Pro4-Luc2 using a specific shRNA (shNOTCH1) and assessed resulting NOTCH1 levels by western blot and reporter assay (Supplementary Figures 4A,B). NOTCH1 knockdown led to a decreased proliferation rate compared to cells transduced with non-targeting shRNA lentivirus ($p < 0.05$ at 48 h and at 72 h) (**Figure 5D**). Notably, we observed that that shNOTCH1-cells display decreased levels of JAG1 mRNA (Supplementary Figure 4C) relative to control cells and that stimulation of shNOTCH1-cells with BMP9 rescued their proliferation rate (**Figure 5D**). To verify the effect of BMP9 on NOTCH signaling activation in our cancer model, we used qRT-PCR to quantify the expression of *JAG1* after BMP9 stimulation in presence of ALK1Fc or CFc. Our transcriptional analysis showed that BMP9 and BMP9+CFc induce mRNA expression of *JAG1* and that ALK1Fc treatment reduces this induction (**Figure 5E**). These data reinforce the hypothesis that the BMP9/ALK2 pathway can drive activation of NOTCH signaling linking two pathways that are associated with tumor progression in prostate cancer.

ALK1Fc Reduces Tumor Burden in the BM18 Patient Derived Xenograft Model

While cell lines and mouse models are of great help in addressing biological questions, there is an increased need for personalized treatments and precision medicine based on screening of human material is becoming increasingly important. Therefore, we tested the antitumoral effect of ALK1Fc on the human patient derived xenograft (PDX) BM18. This PDX was derived

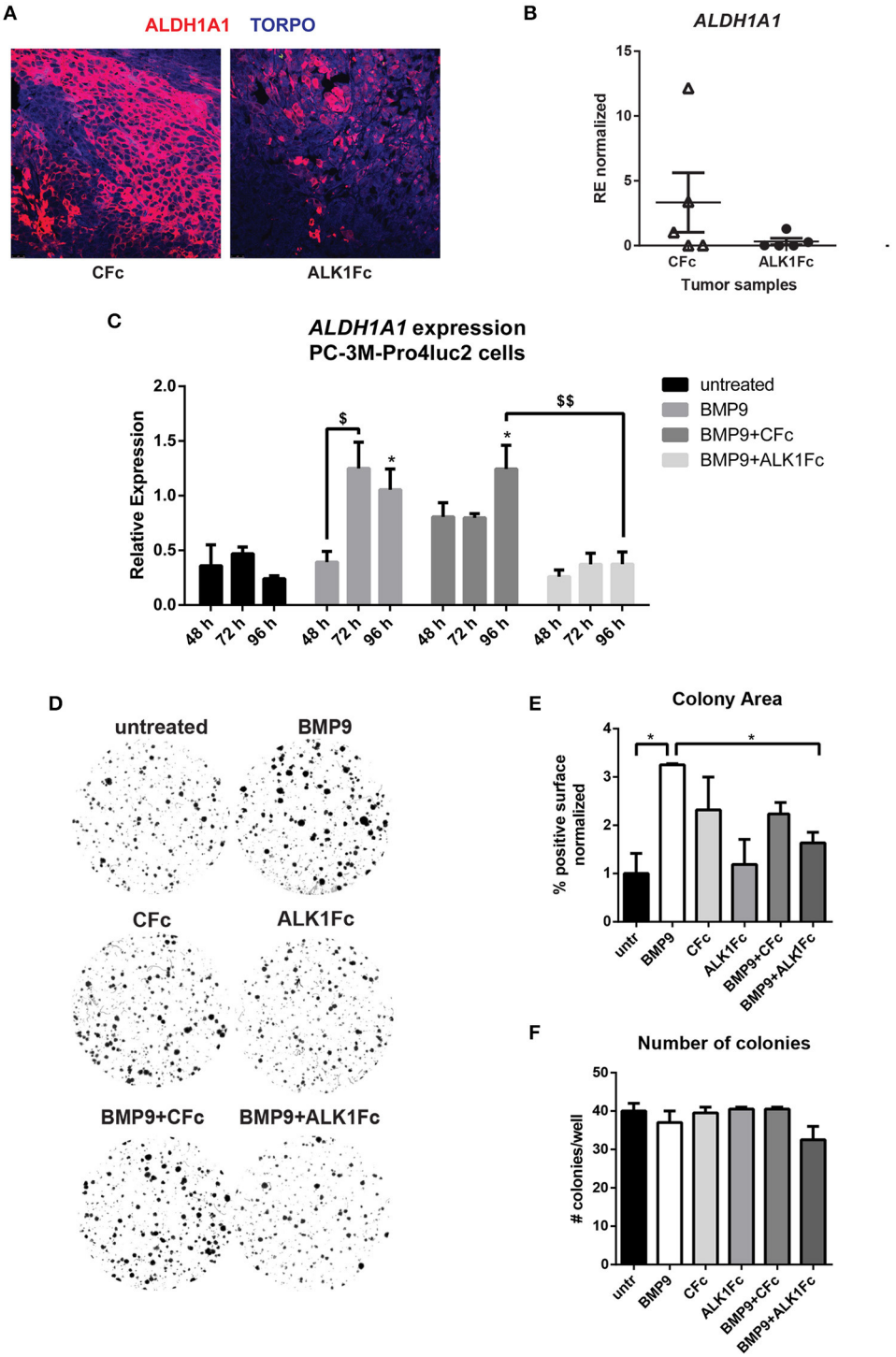

FIGURE 4 | ALK1Fc treatment reduces ALDH1A1 expression. **(A)** Representative images of ALDH1A1 immunofluorescence in prostate tumor samples from ALK1Fc and CFc treated animals. ALDH1A1: red; TOPRO: blue. **(B)** Quantification of ALDH1A1 mRNA by Q-PCR in tumor samples of each group ($n = 5$ for CFc, $n = 5$ for ALK1Fc). **(C)** Expression of *ALDH1A1* in PC-3M-Pro4-Luc2 cells. Relative mRNA expression was measured by Q-PCR from cDNA obtained from PC-3M-Pro4-Luc2 cells treated with BMP9, BMP9+ALK1Fc, BMP9+CFc, for 48, 72, and 96 h. Values are normalized to β-actin expression. Error bars are ± SEM ($n = 3$). $^{\$}P$-value < 0.05; $^{\$\$}P$-value < 0.01. **(D)** Clonogenic assay of PC-3M-Pro4-Luc2 cells. Low-density cultures (100 cells per well of 6 well plate) were stimulated with BMP9, CFc, ALK1Fc, BMP9+CFc, BMP9+ALK1Fc. Colony formation was assessed after 10 days by crystal violet staining. Representative images are shown. **(E,F)** Quantification of surface covered by crystal violet positive colonies (colony area) and colony number. Graph shows percentage of positive surface normalized per condition (average of three independent experiments). $^{*}P$-value < 0.05. Error bars indicate SEM.

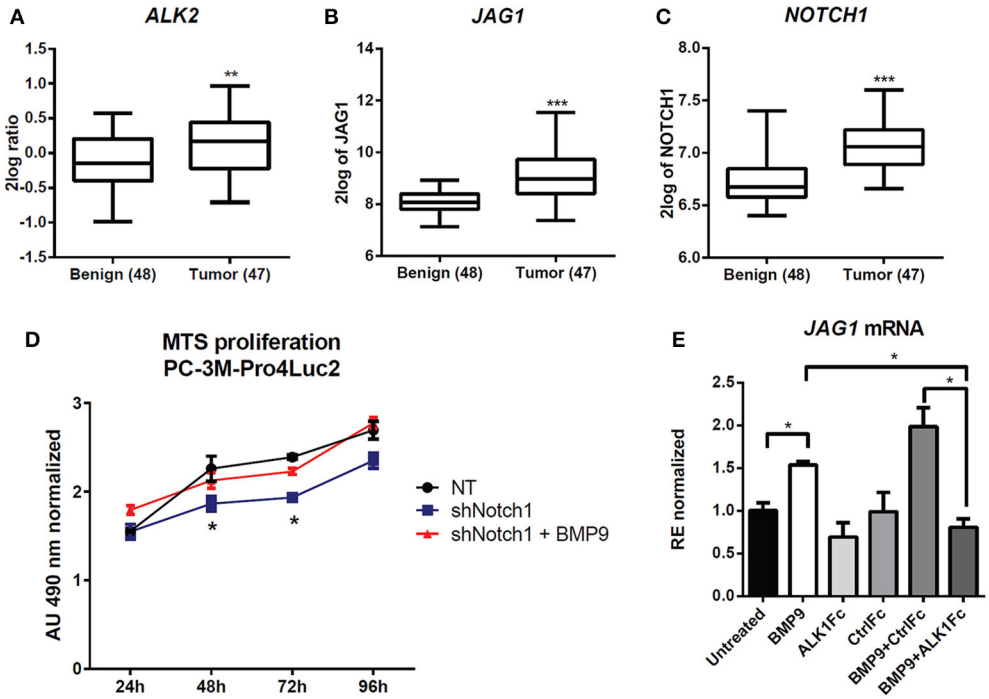

FIGURE 5 | Effect of BMP9 and ALK1Fc on NOTCH signaling pathway. **(A–C)** Bioinformatic analysis of AMC OncoGenomics database (Sueltman transcript comparison) showing mRNA expression of *ALK2* **(A)** *JAG1* **(B)** and *NOTCH1* **(C)** in prostate tissues among benign prostate tissues ($n = 48$) vs. tumor tissues ($n = 47$). Values are expressed as 2log ratio tumor/benign. ***P-value < 0.001. **(D)** MTS assay (24, 48, 72, and 96 h) in PC-3M-Pro4-Luc2 cells transduced with short hairpin RNA against NOTCH1 (shNOTCH1) lentiviral vector or non-targeting (NT) shRNA vector (mock) and plated at low density. BMP9 (1 nM) was added once at cell seeding ($t = 0$). MTS absorbance was measured and values are normalized to the basal measurements $t = 0$ after cell seeding and treatments. Graph represents values for three independent experiments ($n = 3$). Error bars indicate SEM. *P-value < 0.05. **(E)** Expression of *JAG1* in PC-3M-Pro4-Luc2 cells. Relative mRNA expression was measured by Q-PCR from cDNA obtained from PC-3M-Pro4-Luc2 cells treated with BMP9, ALK1Fc, CFc, BMP9+ALK1Fc or BMP9+CFc for 96 h. Values are normalized to β-actin expression. Error bars are ± SEM ($n = 3$). **P-value < 0.01.

from prostate cancer tissue harvested from femoral metastasis (McCulloch et al., 2005) and it is vitally maintained through serial passage in immunocompromised mice (Germann et al., 2012). We transplanted BM18 cells subcutaneously in severe combined immunodeficiency SCID mice and after 1 week the animals were treated with 10 mg/kg ALK1Fc or an IgG control once a week for an additional 5 weeks. Body weights of the mice were monitored weekly (Supplementary Figure 5) and the tumor growth was assessed by caliper measurement. We observed significant reduction in tumor burden upon ALK1Fc but not IgG administration (**Figures 6A,B**) and assessed the prostate epithelial phenotype of the tumors by the hematoxylin eosin (H&E) staining (**Figure 6C**).

DISCUSSION

In this study, we found that BMP9 has a tumor-promoting effect on human prostate cancer cells both *in vitro* and *in vivo*. We demonstrate that blocking BMP9 signaling with ALK1Fc efficiently diminishes prostate cancer cell proliferation and substantially attenuates tumor growth in both an orthotopic model of human prostate cancer and a prostate cancer derived PDX.

BMP9 was first identified in the liver (Song et al., 1995) and active forms are present in serum (Herrera et al., 2009). BMP9 is a ligand for ALK1 in endothelial cells (van Meeteren et al., 2012) and exerts stimulatory or inhibitory effects on endothelial cell growth and migration depending on the cellular context (David et al., 2007; Suzuki et al., 2010; Liao et al., 2017). Aberrant regulation of transforming growth factor-β (TGF-β) and BMP signaling often results in cancer progression (Siegel and Massague, 2003; Massagué, 2008). In particular, BMP ligands, such as BMP9 as well as BMP type I receptors (e.g., ALK1 and ALK2) have been associated with tumor angiogenesis and cancer progression. BMP9 signals through ALK2 in non-endothelial cells including those in ovarian epithelium, where it has been shown to promote proliferation of ovarian cancer cells (Herrera et al., 2009). Similarly, in hepatocellular carcinoma BMP9 has been reported to act as a factor that promotes cell proliferation and survival (Herrera et al., 2013). More recently, the BMP9/ALK2 axis has also been involved in erythroblast cancer cells proliferation (Kim et al., 2017). By contrast, studies have also highlighted the role of BMP9 in reducing breast cancer cell growth and metastasis (Wang et al., 2011, 2017; Ren et al., 2014a,b). Overall, the role of BMP9 and ALKs in promoting or suppressing different cancer types remains controversial and the

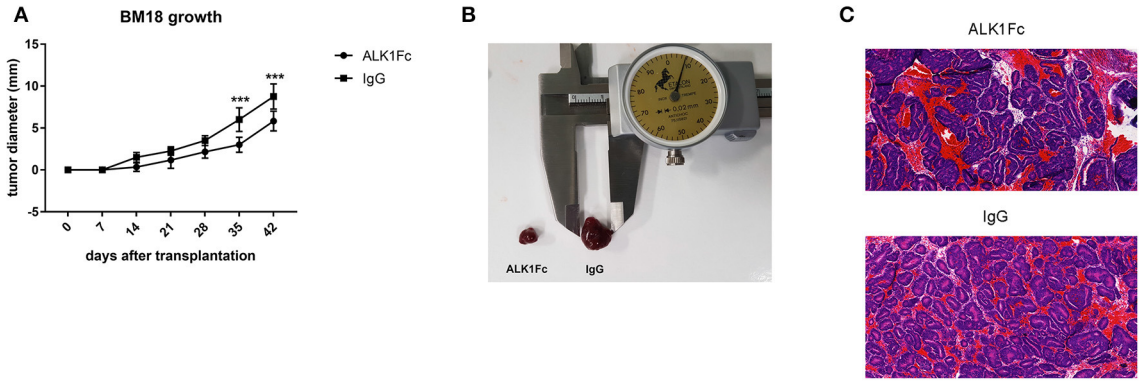

FIGURE 6 | ALK1Fc reduces tumor burden in human prostate cancer xenograft BM18. Human PDX BM18 were transplanted subcutaneously in CB17 SCID mice (two tumors/animal) and treated either with ALK1Fc ($n = 3$) or IgG ($n = 2$). **(A)** Quantification of tumor burden by caliper measurement. Tumors treated with ALK1Fc $n = 6$; tumors treated with IgG $n = 4$. $^{***}P < 0.01$. Error bars indicate ± *SD*. **(B)** Representative images of BM18 tumor size after 5 weeks of treatment. **(C)** HE staining of BM18 after 5 weeks of treatment with ALK1Fc or IgG, left and right respectively.

effect of BMP9 on tumor promotion vs. tumor suppression is likely to be context and cancer-type specific. This provided the rationale for us to elucidate the role of BMP9 in prostate cancer, for which no information is available to our knowledge.

In our search of publicly available databases of human prostate cancer specimens we found that BMP9 was expressed at significantly higher levels in high risk prostate cancer patients compared to the low risk group and that ALK2 was significantly upregulated in malignant vs. benign tissue samples. These data are consistent with our model in which the tumor-promoting effects of BMP9 are mediated by ALK2. Additionally, microarray analysis of data from mouse prostate intraepithelial neoplasia (PIN) vs. invasive cancer in a multistage model of prostate carcinogenesis showed up regulation of ALK2 and BMP9 at the invasive stage in the stromal compartment (Bacac et al., 2006). These data, together with the anti-tumorigenic effect of ALK1Fc documented here, suggest a tumor-promoting role of BMP9 during prostate cancer progression.

Our *in vitro* findings strengthen the afore-mentioned expression data and suggest that BMP9 increases proliferation of human prostate cancer cells. Moreover, our studies with the ALK2 inhibitor LDN193189 support the notion that ALK2 is critically involved in mediating BMP9-induced proliferation in PC-3M-Pro4-Luc2 cells. As depicted in the Results and Supplementary Data sections, treatment with ALK1Fc or LDN193189 alone did not affect proliferation of human prostate cancer cells suggesting a paracrine effect of stroma-derived BMP9 on tumor cells.

We also used an orthotopic model of prostate cancer to demonstrate that ALK1Fc reduces prostate tumor burden and vascular density compared to the controls. Lectin distribution appeared to be less diffuse in ALK1Fc treated animals, suggesting an effect on vessel maintenance rather than angiogenesis. Strikingly, ALK1Fc treatment of tumor-bearing animals resulted in highly hypoxic tumors with a trend in decreased number of CD31+ tumor capillaries suggesting that ALK1Fc may block BMP9-induced neovascularization.

As expected, areas of tumor proliferation and apoptosis were found to be mutually exclusive in their distribution. Apoptotic regions overlapped with hypoxic areas, suggesting that blockade of BMP9 by ALK1Fc might have an effect on proliferation and apoptosis of human prostate cancer cells in addition to targeting vessel maintenance (Mitchell et al., 2010).

SMAD1 and SMAD5 are downstream intracellular effectors of BMP9 signaling and can directly interact with the JAG1 promoter and induce transcription of the NOTCH ligand JAG1 (Larrivee et al., 2012) following BMP9 treatment (Morikawa et al., 2011). Transcriptional analysis revealed that ALK1Fc systemically blocks the induction of JAG1 mRNA in the presence of BMP9 (Morikawa et al., 2011) supporting our hypothesis that the crosstalk between BMP9 and NOTCH signaling may have clinical implications in prostate cancer. Indeed, *in silico* analysis of a previously published dataset of human prostate cancer specimens confirms that both *NOTCH1* and *JAG1* are upregulated at the tumor stage (Borno et al., 2012). In particular, NOTCH signaling seems to promote epithelial-mesenchymal transition in prostate cancer cells (Zhang et al., 2017). Moreover, recent publication shows how NOTCH pathway inhibition antagonizes the growth and invasion of TMPERSS2-ERG positive prostate cancer cells (ERG overexpressing prostate tumor) (Kron et al., 2017) suggesting an important role of the cascade in tumor growth.

Interestingly, NOTCH activates ALDH1A1, an established marker for highly tumorigenic prostate cancer stem cell-like cells (Ginestier et al., 2007; Le Magnen et al., 2013; Zhao et al., 2014; Harris and Kerr, 2017). The ALDH1A1 subpopulation contributes to both tumor initiation and progression and when highly expressed in advanced-stage cancers correlates with poor survival in hormone-naïve patients (Le Magnen et al., 2013). Notably, we show here that ALK1Fc-treated tumors showed significant reduction of ALDH1A1. Taken together, these data suggest that ALK1Fc might potentially interfere with NOTCH signaling in the regulation of ALDH1A1.

Our conclusion that BMP9 promotes aggressive prostate cancer growth was further supported by our demonstration that administration of ALK1Fc inhibited the growth of BM18 PDX, an androgen-dependent bone metastatic prostate tumor. Importantly, these data confirm the ability of ALK1Fc to treat a tumor derived from human patient and open new perspectives in the clinical application of this ligand trap for the cure of prostate cancer.

Our findings provide novel information on the role of BMP9 in human prostate cancer and suggest the promising use of BMP9 targeting molecules for the treatment of tumor and supportive microenvironment in prostate cancer patients.

AUTHOR CONTRIBUTIONS

LA, EZ, and SK: designed the work, acquired and interpreted the data and drafted the manuscript; PG interpreted the data and drafted and revised the manuscript; IK and JG acquired the data and revised the manuscript; MG, LH, GvdP, MS, GT interpreted the data and revised the manuscript; PtD conceived the work, interpreted the data and revised the manuscript; MK conceived the work, acquired and interpreted the data, drafted and revised the manuscript. All authors approved the version to be published and are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2017.00104/full#supplementary-material

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Long Non-coding RNAs in Prostate Cancer with Emphasis on Second Chromosome Locus Associated with Prostate-1 Expression

*Alessia Cimadamore[1†], Silvia Gasparrini[1†], Roberta Mazzucchelli[1], Andrea Doria[1], Liang Cheng[2], Antonio Lopez-Beltran[3], Matteo Santoni[4], Marina Scarpelli[1] and Rodolfo Montironi[1]**

[1] Section of Pathological Anatomy, School of Medicine, Polytechnic University of the Marche Region, United Hospitals, Ancona, Italy, [2] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [3] Department of Pathology and Surgery, Faculty of Medicine, Universidad de Córdoba, Córdoba, Spain, [4] Oncology Unit, Macerata Hospital, Macerata, Italy

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Caterina Nardella,
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Reviewed by:
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Central University of Rajasthan, India

***Correspondence:**
Rodolfo Montironi
r.montironi@univpm.it

[†]These authors have contributed equally to this work.

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Long non-coding RNAs (lncRNAs) are a class of RNA with transcripts longer than 200 nucleotides that lack functional open reading frames. They play various roles in human carcinoma, such as dysregulating gene expression in prostate cancer (PCa), which results in cancer initiation, development, and progression. The non-coding RNA SChLAP1 (second chromosome locus associated with prostate-1) is highly expressed in approximately 25% of PCas with higher prevalence in metastatic compared to localized PCa. Its expression is detectable non-invasively in PCa patient urine samples. Experimental data suggest that targeting SChLAP1 may represent a novel therapeutic application in PCa. This contribution focuses on the role of lncRNAs SChLAP1 expression in PCa diagnosis and prognosis.

Keywords: second chromosome locus associated with prostate-1, metastatic prostate cancer, long non-coding RNA, lethal prostate cancer, prognostic biomarker, marker of aggressiveness

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second major cause of cancer death in man (1). It is characterized by a wide and heterogeneous spectrum of clinical behaviors, ranging from indolent to aggressive forms. The clinical and morphological methods and features currently used in the routine show a low-predictive value concerning the definition of its level of aggressiveness (2, 3). Predictive and prognostic markers can be determined with clinical and pathological parameters, such as serum prostate-specific antigen (PSA), Gleason score (GS), and TNM stage. Due to PCa heterogeneity, patients with similar TNM stage, GS, and PSA could show opposite outcomes (4).

Additional predictive and prognostic markers are needed to distinguish high-risk from low-risk PCa patients. To this end, genetic and epigenetic investigations have been made to understand the complex genomic landscape of PCa in order to improve its diagnosis and prognosis and to define the potential role of new therapeutical targets (5). Long non-coding RNAs (lncRNAs), a class of RNA with transcripts longer than 200 nucleotides without functional open reading frames, play various roles in human carcinoma (6, 7).

This contribution focuses on the role of lncRNAs second chromosome locus associated with prostate-1 (SChLAP1) expression in PCa diagnosis and prognosis.

LONG NON-CODING RNAs

Long non-coding RNAs are a class of RNA with transcripts longer than 200 nucleotides that lack functional open reading frames (6). Based on their locations in the genome relative to protein-coding genes, lncRNAs have been subdivided into intergenic and intragenic. Intragenic lncRNAs can be further subclassified as exonic, intronic, and overlapping lncRNAs (8). Since protein-coding genes have been the focus of most research, the functional role of lncRNAs has been either underestimated or neglected (9).

As shown by Rinn and Chang, "More than 90% of human genome transcripts, including lncRNAs, do not code for proteins" (10). However, accumulating evidence suggests that lncRNAs play a role in the development of various types of cancers, such as PCa, hepatocellular carcinoma, non-small cell lung cancer, leukemia, colon carcinoma, and breast cancer (11–16).

While the mechanism of many lncRNAs remains to be elucidated, it has become clear that lncRNAs contribute to dysregulation of gene expression in PCa, thus resulting in cancer initiation, development, and progression (17).

lncRNAs IN PROSTATE CANCER

Elucidating the roles of lncRNAs in PCa holds great promise for early detection, prevention, and treatment. A well-known example is prostate cancer antigen3 (PCA3), also known as DD3, initially discovered *via* expression profiling of prostate sample (18). PCA3 has been extensively studied as a PCa-specific biomarker in body fluids. PCA3 urine RNA assay predicts biopsy status and histopathological characteristics (19). However, it does not predict outcomes such as recurrence and metastasis.

Another lncRNA investigated in PCa is metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1), originally known to be overexpressed in patients at high risk for non-small cell lung cancer metastasis, as its name implies (20, 21). Its expression is found in many other human solid tumors having close correlation with invasiveness and metastasis (22–26). Ren and colleagues found that MALAT-1 is overexpressed in PCa compared to adjacent normal tissue (20). MALAT-1 expression is significantly higher in castration-resistant PCa (CRPCa) than in primary prostate tumor. Its expression increases from hormone sensitive to CRPCa. The same group of authors showed that this lncRNA could be a promising therapeutic target in patients with CRPCa. The intratumoral administration of therapeutic MALAT-1 siRNA suppressed CRPCa growth and metastasis *in vivo*, and prolonged the survival of tumor bearing mice (20).

It has also been shown that urine MALAT-1 is an independent predictor of PCa, more accurate than routine PSA. Its use would prevent one-third of unnecessary biopsies in PSA 4–10 ng/ml cohorts, without missing any high-grade PCa (27). Furthermore, in 192 plasma samples, MALAT-1 achieved high diagnostic accuracy in predicting prostate biopsy outcomes and, therefore, it might also be utilized as a plasma-based biomarker for PCa detection (28).

Recently, Zhao et al. investigated the expression profile of FALEC, another lncRNA, in PCa. Like other lncRNAs, its expression is significantly higher in PCa than adjacent normal parenchyma. Its downregulation inhibits cell proliferation, migration, and invasion (29).

Similar results were obtained with CCAT2, a lncRNA involved in proliferation, migration, and invasion of PCa cells. In particular, it was demonstrated that silencing of CCAT2 was able to inhibit N-cadherin, vimentin expression, and improve the expression level of E-cadherin, thus leading to the stimulation of epithelial-mesenchymal transition. High expression level of CCAT2 correlates with poor overall survival and progression-free survival and could be considered an independent prognostic factor in patients with PCa (30). Another promising lncRNA is LOC400891 which showed high expression in patients with an advanced PCa and a shorter biochemical recurrence-free survival time (31).

An interesting feature of lncRNAs is that many of them are not PCa specific. For example, we can observe overexpression of lncRNA-ATB in gastric cancer (32), hepatocellular carcinoma (33), osteosarcoma (34), and other tumors. Its tissue overexpression is directly proportional with the histological grade, high preoperative PSA level, pathological stage, high GS, lymph node metastasis, angiolymphatic invasion, and biochemical recurrence in PCa patients (35).

lncRNA-Based Signature

Signatures, based on microarray lncRNA expression profiling, have been recently developed and widely used in prediction of a series of tumor characteristics and outcomes in various cancer type (36, 37). However, due to its low expression characteristics, a single lncRNA analysis might be associated with false-positive result. To overcome this problem, Huang and colleagues have developed a risk score based on lncRNA expression profile (38). They found four lncRNAs are significantly associated with BCR-free survival. Among the four lncRNAs, two (RP11-108P20.4 and RP11-757G1.6) were positively associated with BCR-free survival, while the remaining two (RP11-347I19.8 and LINC01123) were negatively associated with BCR-free survival. They estimated a risk score for each patient and then divided patients into a high-risk group and a low-risk group by using the median risk score as the cutoff point. The four-lncRNA signature has been shown to be a powerful prognostic factor, independent of age, tumor and lymph node status, GS, margin status, and adjuvant postoperative radiotherapy (38) in (**Table 1**).

SECOND CHROMOSOME LOCUS ASSOCIATED WITH PROSTATE-1

About 1,800 lncRNAs were identified by Presner et al. through the application of RNA sequencing techniques (i.e., transcriptome sequencing) on a consistent number of tissue samples. Of these 1,800 lncRNAs, 121 resulted transcriptionally dysregulated in PCa (39). Such 121 Prostate Cancer-Associated Transcripts

TABLE 1 | Other lncRNAs associated with prostate cancer.

PCA3	Urine marker useful to predict biopsy status and histopathological characteristics (19)
MALAT-1	Its expression increases from hormone sensitive to CRPCa (20). Useful plasma biomarker for PCa detection (28)
FALEC	Its inhibition decreases cell proliferation, migration, and invasion (29)
CCAT2	Its high expression levels correlates with poor overall survival and progression-free survival (30)
LOC400891	Its high expression correlates with shorter BCR-free survival time (31)
ATB	Its high expression correlates with preoperative PSA levels, pathological stage, GS, lymph node metastasis, angiolymphatic invasion, and BCR (35)
RP11-108P20.4	Positively associated with BCR-free survival
RP11-757G1.6	Part of the Four-lncRNA signature (38)
RP11-347I19.8	Negatively associated with BCR-free survival
LINC01123	Part of the Four-lncRNA signature (38)

CRPCa, castration resistant; PCa, prostate cancer; BCR, biochemical recurrence; PSA, prostate-specific antigen; GS, Gleason score; lncRNAs, long non-coding RNAs.

represent an unbiased list of potentially functional lncRNAs associated with PCa. By performing a cancer outlier profile analysis to identify intergenic lncRNAs selectively upregulated in a subset of cancers, they found two lncRNAs, PCAT-109 and PCAT-114, both located on Chromosome 2q31.3 in a "gene desert," a region of the genome that are lacking of protein-coding genes. Both genes showed "outlier profiles and ranked among the best outliers in PCa" (40). In particular, PCAT-114 was found to be overexpressed in prostate cell lines. It was named SChLAP1 after its genomic location (40). *Schlap1* gene has a transcript length of 24,484 Kb. The complete gene is composed of 7 exons and 1,675 nucleotides. The primary transcript (isoform 1) is composed of 5 exons with a length of 1,436 nucleotides. As a result of a spicing process, a total of 8 isoforms were found, with isoform 1, isoform 2, and 3 accounting for >90% of transcripts (41). RNA-seq, performed on 27 different tissue samples from 95 human individuals, showed that SChLAP1 expression was highly specific for prostate tissue, being present at minor levels in bladder, kidney, and testis samples (42).

SChLAP1's Working Mechanisms: Interaction with SWI/SNF Complex and miR-198

In vitro and *in vivo* gain-of-function and loss-of-function experiments have shown that SChLAP1 plays a crucial role in cancer cell invasiveness and metastasis, antagonizing the activity of the SWI/SNF chromatin-modifying complex, a multiprotein system able to move nucleosomes at gene promoters. In particular, such experiments showed that the inactivation of SWI/SNF complex promoted cancer progression and that multiple SWI/SNF components were somatically inactivated in cancer (41, 43). As shown by Prensner, even though "other lncRNAs, such as HOTAIR and HOTTIP, are known to assist epigenetic complexes such as PRC2 and MLL by facilitating their genomic binding and enhancing their functions, SChLAP1 is the first lncRNA that impairs a major epigenetic complex with well-documented tumor suppressor function" (41, 44).

In vivo, SChLAP1 has been shown to be implicated in tumor cell proliferation and metastasization, as evidenced by both the reduction of tumor growth kinetics and the decreased number and dimensions of metastatic sites as a consequence of the intracardiac injection of 22Rv1 cells with SChLAP1 knockdown in CB-17 SCID mice (41).

Recent studies have shown interaction between SChLAP1 and miR-198. MiR-198 is downregulated in many cancers, such as gastric cancer, lung cancer, and hepatocellular carcinoma (45–47). miR-198 suppress the proliferation and invasion of colorectal carcinoma (48). miR-198 might exert its anticancer effect through inhibition of MAPKs signaling pathway (49). In PCa tissue, a low expression of miR-198 was found. As shown by Li et al., "knockdown of SChLAP1 significantly increased the expression of miR-198 and SChLAP1 overexpression markedly decreased it. Thus, SChLAP1 acted as a negative regulator in the expression of miR-198" and subsequently modulated the MAPK1 signaling pathway in PCa (49) (**Figure 1**). Transfecting PCa cells with a designed-specific siRNA to knockdown SChLAP1 expression, investigators have obtained, as expected, a significantly reduction in cell proliferation together with an increase in apoptosis-associated proteins. Furthermore, SChLAP1 knockdown determined a decrease of MMP-9, MMP-14, and VEGF expressions both *in vitro* and *in vivo*, confirming its involvement in cancer invasiveness and metastasis (49). All such findings show multiple interactions between SChLAP1 and factors involved in oncogenesis and cancer progression and explain the mechanism

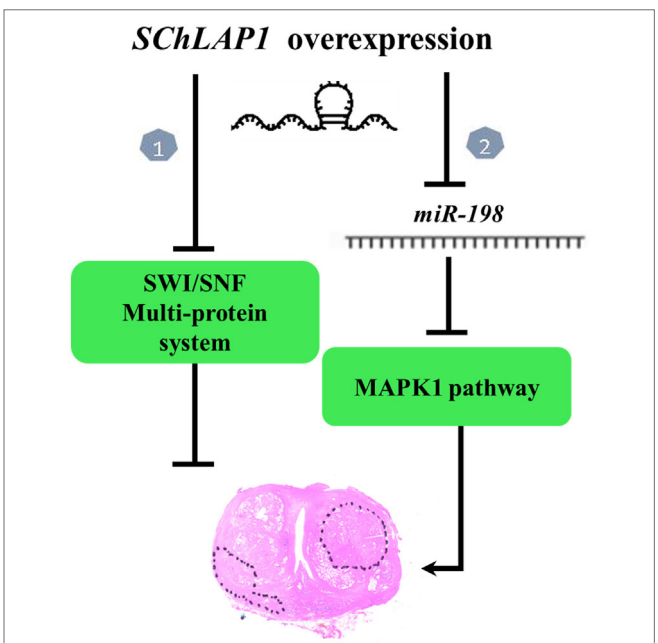

FIGURE 1 | Working model of second chromosome locus associated with prostate-1 (SChLAP1) in prostate cancer (PCa). There are two pathways. In Pathway 1, loss of SWI/SNF functionality promotes cancer progression (41). In Pathway 2, miR-198 might exert its anticancer effect through inhibition of MAPKs signaling pathway. SChLAP1 acts as a negative regulator in the expression of miR-198 and subsequently modulates the MAPK1 signaling pathway in PCa (49).

through which SChLAP1 promotes migration and invasion of PCa (50). Understanding this molecular pathway is essential for exploring new potential strategies for early diagnosis and therapy.

SChLAP1: Predictor of Aggressive PCa

Second chromosome locus associated with prostate-1 is highly expressed in approximately 25% of PCa, expression being higher in metastatic compared to localized prostate cancers. It was associated with ETS gene fusions (41). Multivariate and univariate regression analyses have demonstrated that SChLAP1 expression is an independent predictor of PCa aggressiveness with highly significant hazard ratios for predicting BCR, clinical progression to systemic disease, and PCa-specific mortality, compared to other clinical factors such as advanced clinical stage and the GS (41). Moreover, SChLAP1 expression was significantly associated with extracapsular extension, seminal vesicle invasion, and positive surgical margin status (40).

Validation in three-independent cohorts has confirmed the prognostic value of SChLAP1 for metastasis. On multivariate modeling, SChLAP1 expression independently predicted metastasis within 10 years, death within 10 years, and biochemical recurrence within 5 years with odds ratios, comparable to GS. Among all known genes, the lncRNA SChLAP1 ranked first for elevated expression in patients with metastatic progression by receiver-operator-curve area-under-the-curve analyses and was the only prostate-specific gene, ideal for development as a non-invasive biomarker (40).

Similar results have been obtained by Mehra and colleagues using a novel RNA *in situ* hybridization (ISH) assay for detection of SChLAP1 in formalin-fixed, paraffin-embedded tissue (51). They found that high SChLAP1 expression independently predicts biochemical PCa recurrence after radical prostatectomy in patients with clinically localized PCa and that it is associated with the development of lethal PCa.

Interestingly, high SChLAP1 expression is associated with lethal PCa among patients with non-advanced clinical tumor stage. Similarly, high SChLAP1 expression is associated with lethal PCa among patients with low grade tumors (GS $\leq$7). However, using SChLAP1 as prognostic test, investigators have obtained a sensitivity around 24% and a specificity of 94% in the group of non-advanced clinical tumor stage and in the group with a 6–7 GS (52). Considering the low sensitivity of the test in the identification of an aggressive disease in contrast with low-risk morphological features, the evaluation of SChLAP1expression alone does not seem to improve treatment decision. In conjunction with other prognostic tools, SChLAP1 has been shown to improve upon established clinical algorithms for the risk stratification of PCa patients, specifically the CAPRA-S score (53, 54), i.e., one of the best clinic-pathological models to date. SChLAP1 further improves prediction upon both the Decipher test 50 and the CCP gene signature (55, 56).

Recently, Chua et al. have investigated SChLAP1 expression in subsets of PCa characterized by cribriform architecture (CA) and intraductal carcinoma (IDC), features both associated with increased risks of biochemical relapse and metastasis. Besides the histological presentation, IDC/CA+ cancers harbor an increased percentage of genome aberration (PGA). This is in agreement with the observation that tumors with IDC or genomic instability have a greater metastatic potential. Using mRNA abundance analyses and assessing >25,000 genes, they found that SChLAP1 was surprisingly the only gene with more than threefold higher expression in IDC/CA+ compared to IDC/CA− cancers (57).

The association of SChLAP1 expression within IDC/CA+ tumors has also been further demonstrated by SChLAP1 RNA-ISH in prostatectomy TMA-cores. The SChLAP1+, IDC/CA+ subgroup has shown a significant increase of biochemical relapse, independent of PGA. Combining histology features of cribriform architecture and intraductal carcinoma with genomic instability or SChLAP1 expression can stratify patients for recurrence more accurately than any parameter alone. Interestingly, SChLAP1 RNA-ISH diffuse expression has been observed in the cribriform architecture and intraductal carcinoma and in the adjacent invasive adenocarcinoma. This further supports a field defect and a common clonal ancestor to both histopathologies (58).

SChLAP1 in Urine Sediments

Second chromosome locus associated with prostate-1 expression is detectable non-invasively in PCa patient in urine samples. Its expression is both more frequent and more highly elevated in GS 7 compared to GS 6 patients even if it is less sensitive than PCA3 and TMPRSS-ERG gene fusion (40). "SChLAP1 expression may complement existing urine diagnostic assays, including PCA3 and TMPRSS2-ERG, and that clinical application of a SChLAP1 urine test would be most effective in conjunction with these, and potentially other, urine assays" (59).

SChLAP1 As a Potential Drug Target

RNA interference (RNAi) technology using short interfering RNA (si-RNA) has shown great potential in the treatment of cancers through silencing of specific genes. *In vitro* and *in vivo* experiments have demonstrated that SChLAP1-knockdown promoted apoptosis and inhibited cell proliferation and invasion (41, 49). SChLAP1 is also overexpressed in bladder cancer compared to paired normal bladder tissues. Cell transfected with SChLAP1 siRNA showed growth arrest, apoptosis, and migration inhibition, suggesting oncogenic roles in bladder cancer and a potential therapeutic target (**Table 2**) (60). Such results might be the starting point to investigate the therapeutical potential of antagonizing SChLAP1 oncogenic functions (60).

lncRNA IN DIAGNOSIS, PROGNOSIS, AND TREATMENT: PROS AND CONS

There are *Pros* and *Cons* with the use of lncRNA in the diagnosis and treatment of PCa patients. Indeed, lncRNA may represent a useful biomarker that can give to clinicians fundamental information on tumor biological behavior and aggressiveness, leading to the possibility of designing personalized and tailored strategies for a single PCa patient. This may also allow an optimization of patients' outcome and to avoid useful costs and consequences of not effective therapies for PCa patients.

As far as the *Cons*, tumor aggressiveness is the result of a complex process that involves lncRNA and a variety of driver

TABLE 2 | SChLAP1 expression in prostate cancer.

Reference	Result	Method	No. specimens
Prensner (39)	• 121 novel lncRNA loci (out of >1,800) were aberrantly expressed in PCa tissues • Only two, PCAT-109 and PCAT-114, showed striking outlier profiles and ranked among the best outliers in PCa	RNA-Seq; COPA	102 PCa tissue samples and cell lines
Prensner (41)	• SChLAP1 expression is an independent predictor of PCa aggressiveness with highly significant hazard ratios for predicting BCR, CP, and PCSM • SChLAP1 antagonizes tumor-suppressive functions of the SWI/SNF complex	qPCR	235 RP localized PCa
Prensner (40)	• SChLAP1 expression independently predicted metastasis, PCa-specific death, and BCR with OR comparable to GS • SChLAP1 expression was detectable non-invasively in urine samples and associated with higher-risk patients	RNA extraction, microarray hybridization; qPCR	1,008 patients. Three independent cohorts; 230 urine sediment samples
Mehra (51)	• SChLAP1 expression is enriched in samples from tumors with high GSs (≥8) compared to tumors with lower GSs • High SChLAP1 expression independently predicts BCR (PSA relapse) after RP	ISH	160 clinically localized PCa
Mehra (52)	• High SChLAP1 expression is significantly associated with a higher risk of lethal PCa and PCa-specific death independent of age at diagnosis, GS, and pathologic stage • High SChLAP1 expression is associated with lethal PCa among patients with non-advanced clinical tumor stage, but not among patients with advanced clinical tumor stage	ISH	937 PCa patients
Zhang (60)	• SChLAP1 was overexpressed in bladder cancer tissues compared to paired normal bladder tissues • Cell growth arrest, apoptosis induction, and migration inhibition were also observed in bladder cancer cells after transfection with SChLAP1 siRNA	qPCR; CCK-8 assay, flow cytometry analysis, and wound healing assay	Bladder cancer T24 and 5,637 cells
Chua (57)	• SChLAP1 was the only gene expressed at >3-fold higher in intraductal carcinoma (IDC) and cribriform architecture (CA) PCa than in IDC/CA - tumors	Profiling of mRNA abundance, ISH	1,325 localized PCa
Li (49)	• Knockdown of SChLAP1 promoted apoptosis and inhibited cell proliferation and invasion *in vitro* and *in vivo* • SChLAP1 acted as a negative regulator in the expression of miR-198 and accelerates the proliferation and metastasis of PCa promoting the MAPK1 pathway	Not available	Not available

RNA-Seq, next generation transcriptome sequencing; COPA, cancer outlier profile analysis; PCa, prostate cancer; qPCR, quantitative Polymerase Chain Reaction; FFPE, formalin-fixed paraffin embedded; BCR, biochemical recurrence; CP, clinical progression; PCSM, prostate cancer-specific mortality; GS, Gleason score; OR, odds ratio; RP, radical prostatectomy; ISH, in situ hybridization; SChLAP1, second chromosome locus associated with prostate-1; lncRNAs, long non-coding RNAs.

genes, leading to the necessity for uropathologists to test and validate not a single driver gene, but a panel of genes with direct consequences on the relative costs of these procedures.

CONCLUSION

In conclusion, research on lncRNAs in PCa is at its onset. However, as shown in this review:

- The first set of data has revealed central roles with clinical significance for lncRNAs in different stages of the disease.
- There is evidence that lncRNAs, including SChLAP1, are critical in PCa development and progression.
- Concerning future perspective, mainly based on experimental data, targeting SChLAP1 may become a novel therapeutic application in PCa.

AUTHOR CONTRIBUTIONS

RM: conception and design. AC and SG: drafting the manuscript. AD and RMa: acquisition of data. LC and AL-B: critical revision of the manuscript. MS: supervision.

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microRNAs Make the Call in Cancer Personalized Medicine

*Simone Detassis, Margherita Grasso, Valerio Del Vescovo and Michela A. Denti**

Laboratory of RNA Biology and Biotechnology, Centre for Integrative Biology, University of Trento, Trento, Italy

Since their discovery and the advent of RNA interference, microRNAs have drawn enormous attention because of their ubiquitous involvement in cellular pathways from life to death, from metabolism to communication. It is also widely accepted that they possess an undeniable role in cancer both as tumor suppressors and tumor promoters modulating cell proliferation and migration, epithelial-mesenchymal transition and tumor cell invasion and metastasis. Moreover, microRNAs can even affect the tumor surrounding environment influencing angiogenesis and immune system activation and recruitment. The tight association of microRNAs with several cancer-related processes makes them undoubtedly connected to the effect of specific cancer drugs inducing either resistance or sensitization. In this context, personalized medicine through microRNAs arose recently with the discovery of single nucleotide polymorphisms in the target binding sites, in the sequence of the microRNA itself or in microRNA biogenesis related genes, increasing risk, susceptibility and progression of multiple types of cancer in different sets of the population. The depicted scenario implies that the overall variation displayed by these small non-coding RNAs have an impact on patient-specific pharmacokinetics and pharmacodynamics of cancer drugs, pushing on a rising need of personalized treatment. Indeed, microRNAs from either tissues or liquid biopsies are also extensively studied as valuable biomarkers for disease early recognition, progression and prognosis. Despite microRNAs being intensively studied in recent years, a comprehensive review describing these topics all in one is missing. Here we report an up-to-date and critical summary of microRNAs as tools for better understanding personalized cancer biogenesis, evolution, diagnosis and treatment.

Keywords: microRNAs, personalized medicine, cancer, MiR-SNP, biomarker

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Simona Pisanti,
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Chandi C Mandal,
Central University of Rajasthan, India
Botao Zhao,
Shanghai University, China

****Correspondence:***
Michela A. Denti
denti@science.unitn.it

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MICRORNA BIOGENESIS

microRNAs are small non-coding RNAs described for the first time in 1993 (Lee et al., 1993). They are found in plants (Jones-Rhoades et al., 2006), animals and viruses (Grundhoff and Sullivan, 2011), with functions in RNA silencing and post-transcriptional regulation of gene expression. They also have a role in pathological processes including neurodegenerative diseases (Molasy et al., 2016; Reddy et al., 2016) and cancer (da Silva Oliveira et al., 2016; Mohammadi et al., 2016). microRNAs transcriptional units are present both in introns or exons of other genes and as independent ones (Godnic et al., 2013). They are transcribed mainly by RNA Polymerase II, capped and polyadenylated forming primary microRNAs (pri-microRNAs). A small group is generated by RNA Polymerase III. The pri-microRNA is processed in a precursor microRNA (pre-microRNA)—about 70 nt—by RNase III Drosha and RNA-binding protein DGCR8 (Lee et al., 2003). Subsequently, the pre-microRNA is transported out of the nucleus via exportin-GTPase

RAN system, where is further processed by Dicer producing the double-stranded microRNA of 22nt (Wilson et al., 2015). A complex made of AGO proteins is able to bind it and form the miRISC. Only one strand of the microRNA is loaded in the RISC complex, while the other (the passenger strand) is thought to be degraded. The RISC complex has an important post-transcriptional role in gene expression, regulating stability and turnover of mRNAs. The loaded microRNA can target mRNAs, exploiting its sequence complementarity. If the match is perfect the system leads to the mRNA degradation (Yekta et al., 2004), otherwise it impedes its translation (Ipsaro and Joshua-Tor, 2015). Because of their short length, microRNAs, which usually bind the 3′UTR of target mRNAs, are able to target several distinct mRNAs and, on the other hand, any given mRNA may present many binding sites for different microRNAs (Bartel et al., 2009).

MICRORNA AND CANCER

It has been widely reported that microRNAs are involved in many aspects related to cancer (**Figure 1**). Following the "hallmarks" of cancer (Hanahan and Weinberg, 2011) we can find many articles in which microRNAs play a role in each of these steps on the road of cancer biogenesis and progression. Here we describe some examples (**Table 1**).

1. *Sustaining the proliferative signal*. miR-27a-3p was shown to be associated with progression of nasopharyngeal cancer from patient samples and to be increased compared to healthy tissues. *In vitro* it promotes 5–8 F cell proliferation, migration and invasion targeting MAPK10 (Li and Luo, 2017). On the contrary, miR-545 was found decreased in colorectal cancer (CRC) in comparison to normal tissues and thus, its over-expression led to diminished proliferation and colony formation capacity (Huang and Lu, 2017). Luciferase and western blot assay confirmed the *in-silico* prediction of miR-545 targeting EGFR in CRC cell lines.
2. *Evading tumor suppressors*. Liu and colleagues (Liu Y. et al., 2017) showed how miR-19a binds directly the 3′UTR of TIA1 mRNA, involved in stress granuli formation and in the apoptotic pathway, promoting cell proliferation and migration in CRC cells, boosting also tumor growth in xenograft mice.
3. *Resistance to cell death*. It has been reported that miR-29 is an endogenous regulator of MCL-1 protein expression, an anti-apoptotic molecule, and it has been found down-regulated in cholangiocarcinoma cell lines (Mott et al., 2007). Similarly, miR-15a and miR-16-1, found deleted or down-regulated in the majority of chronic lymphocytic leukemias (CLLs), can directly negatively regulate BCL-2 in CLL. Their expression was described as inversely correlated to BCL2 expression in CLL and their over-expression may induce apoptosis in a leukemic cell line model through BCL2 repression (Cimmino et al., 2005).
4. *Enabling replicative immortality*. miR-130b∼301b cluster is hypermethylated in prostate cancer cells and it was demonstrated how its expression restoration can replace senescence mechanisms reducing the malignant phenotype of prostate cancer cells (Chen et al., 2015; Ramalho-Carvalho et al., 2017). Similarly, miR-137 levels are significantly reduced in human pancreatic cancer leading to a defective senescence response. This small non-coding RNA targets KDM4A which expression contributes to avoid miR-137-induced senescence. Therefore, restoration of miR-137 expression it has been reported to promote senescence and dampen proliferation of pancreatic cancer cells (Neault et al., 2016).
5. *Inducing angiogenesis*. miR-135a is generally decreased in gastric cancer tissues compared to normal samples. It targets FAK which is an important regulator and effector of VEGF in tumor angiogenesis. It has been described that upon miR-135a over-expression, the protein levels of FAK in gastric cancer cell lines decrease significantly. Therefore, it has been proposed that miR-135 inhibits tumor growth, migration, invasion and angiogenesis by targeting focal adhesion kinase (FAK) pathway (Cheng et al., 2017). Differently, miR-23 in lung cancer cells under hypoxic conditions is up-regulated in the secretome and directly targets prolyl hydroxylase 1 and 2, enhancing the accumulation of the hypoxia-inducible factor-1α. Consequently, hypoxic lung cancer cells enhanced angiogenesis. In addition, it has been shown how secreted miR-23a also inhibits tight junction protein ZO-1, thereby increasing vascular permeability and cancer trans-endothelial migration. Moreover, inhibition of miR-23a dampened angiogenesis and tumor growth in mice and miR-23a found in sera of lung cancer patients positively correlated with proangiogenic activities (Hsu et al., 2017).
6. *Activation of invasion and metastasis*. Daugaard and colleagues demonstrated via RNA-seq analysis of formalin-fixed paraffin embedded (FFPE) lung adenocarcinomas from patients with and without detectable metastasis disease, that down-regulation of miR-30a-3p and up-regulation of miR-210-3p were significantly associated with the presence of distant metastases (Kumarswamy et al., 2012; Daugaard et al., 2017). Microarray analysis and quantitative PCR by the Law laboratory identified and validated up-regulated miR-885-5p in liver metastases when compared to primary CRCs. Furthermore, over-expression of miR-885-5p *in vitro* led to cell migration, invasion and *in vivo* development of liver and lung metastases. miR-885-5p targets directly the 3′UTR of CPEB2 which negatively regulates TWIST1, a well-known player in epithelial-mesenchymal transition (EMT) (Siu-Chi Lam et al., 2017). Alike, miR-9 may promote ovarian cancer metastasis targeting E-CADHERIN and upregulating N-CADHERIN and VIMENTIN, mesenchymal markers (Zhou et al., 2017).
7. *Reprogramming energy metabolism*. It is well-known that cancer cells are able to modify its metabolism favoring survival and proliferation. miR-7 has been demonstrated to decrease the usually up-regulated metabolic autophagy in pancreatic cancer cells via affecting LKB1-AMPK-mTOR signaling (Gu et al., 2017). Another tumor suppressor microRNA is miR-1 which has been described to be down-regulated in CRC cell lines compared to normal colon epithelial cells. Moreover, over-expression of miR-1 decreases cancer cell proliferation

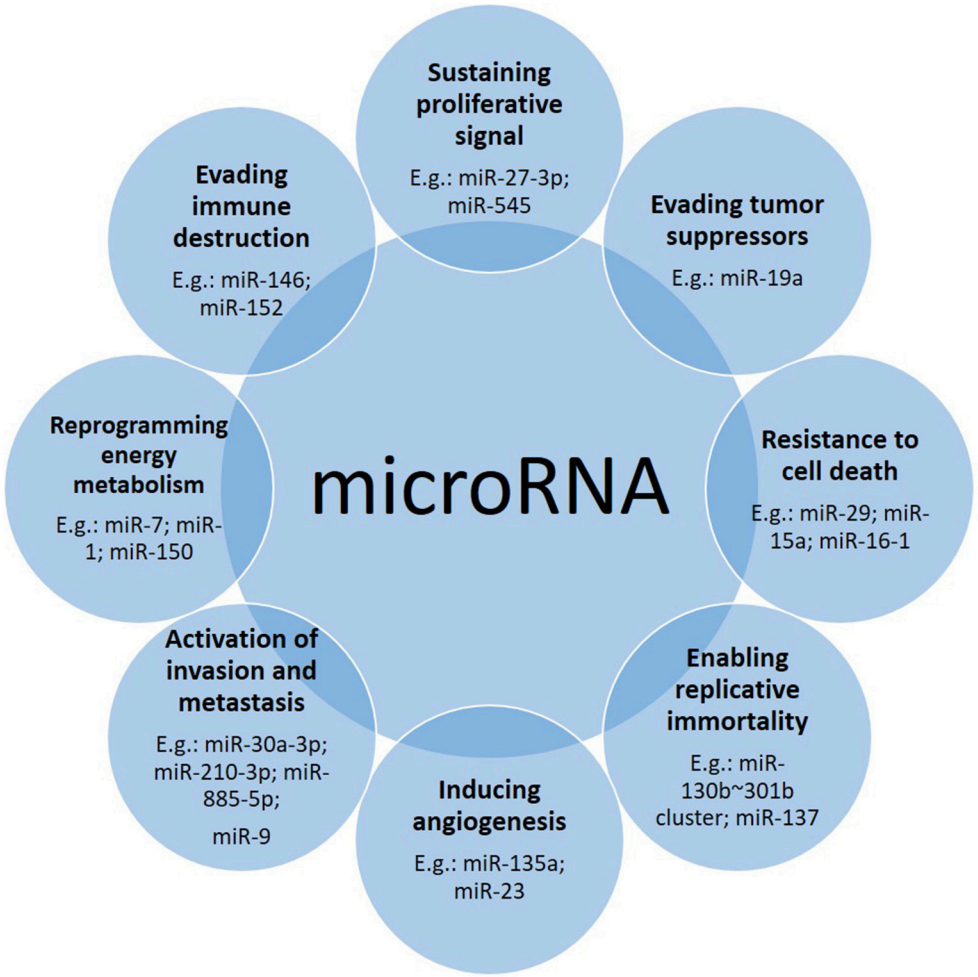

FIGURE 1 | Down- or up-regulation of microRNAs contribute to the cancer driving steps. Often one microRNA affects more than one hallmark, with one prevailing tissue-dependent mechanism.

dampening aerobic glycolysis, lactate production and glucose uptake *in vitro* targeting HIF-1α and impacting SMAD3 pathway (Xu et al., 2017). On the contrary, high levels of miR-150 in glioma cells increased the Warburg effect, via the targeting of VHL 3′UTR, facilitating *in vivo* tumor growth (Li et al., 2017).

8. *Evading immune destruction.* Khorrami and colleagues showed how over-expression of miR-146 in a CRC cell line co-cultured with peripheral blood mononuclear cells extracted from healthy donors, increased T_{reg} frequencies and anti-inflammatory cytokines like TGF-β and IL-10, leading to an overall immune suppression in the tumor microenvironment (Rusca and Monticelli, 2011; Khorrami et al., 2017). On the other hand, miR-152 was shown to be decreased in gastric cancer cell lines as well as in human gastric cancer tissues. Restoration of its expression leads to enhanced T cells proliferation and effector cytokines production through the inhibition of the B7-H1/PD-1 pathway (Wang Y. et al., 2017).

In this network of complexity, it should be added that one microRNA, in view of its target promiscuity, could have multiple roles in different type of cancers. miR-21 is one of those microRNAs. It has been found to be an anti-apoptotic factor in breast cancer (Si et al., 2007) and its suppression increased CASPASE3/7 enzymatic activities in human glioblastoma cells (Chan et al., 2005). Moreover, miR-21 is able to sustain proliferative signal targeting PTEN, a well-known tumor suppressor, in cholangiocarcinoma (He Q. et al., 2013; Wang L.-J. et al., 2015) and human hepatocellular carcinoma (Meng et al., 2007), inhibiting AKT and mTOR pathway which promotes cell survival and proliferation. miR-21 is linked with PI3K/AKT pathway also via the inhibition of FOXO1 in large B-cell lymphoma (Go et al., 2015). It was also shown to target TPM1 which normally is considered a tumor suppressor gene, regulating microfilament formation and anchorage-independent growth in a breast cancer cell line (Zhu et al., 2007). In addition, in breast cancer, miR-21 has been reported to sustain EMT signaling and IL-6 levels

TABLE 1 | Examples of microRNAs involved in the hallmark of cancer.

Hallmark	microRNA	De-regulation in cancer	Target	Function	References
Sustaining proliferative signal	miR-27-3p	↑ in nasopharyngeal cancer	MAPK10	Regulation of ERK1 and ERK2 cascade	Li and Luo, 2017
	miR-545	↓ in colorectal cancer	EGFR	Signaling pathway	Huang and Lu, 2017
Evading tumor suppressors	miR-19a	↑ in colorectal cancer	TIA1	Major granule associated species	Liu Y. et al., 2017
Resistance to cell death	miR-29	↓ in cholangiocarcinoma	MCL-1	Regulation of apoptosis vs. cell survival, and maintenance of viability	Mott et al., 2007
	miR-15a, miR-16-1	↓ in chronic lymphocytic leukemias	BCL-2	Suppresses apoptosis	Cimmino et al., 2005
Enabling replicative immortality	miR-130b∼301b cluster	↓ in prostate cancer	MMP2	Matrix remodeling	Ramalho-Carvalho et al., 2017; Chen et al., 2015
	miR-137	↓ in pancreatic cancer	KDM4A	Histone demethylase	Neault et al., 2016
Inducing angiogenesis	miR-135a	↓ in gastric cancer	FAK	Non-receptor protein-tyrosine kinase	Cheng et al., 2017
	miR-23	↑ in lung cancer	PH1; PH2; ZO-1	Alanine-Glyoxylate Aminotransferase; Glyoxylate And Hydroxypyruvate Reductase; Tight Junction Protein	Hsu et al., 2017
Activation of invasion and metastasis	miR-30a-3p	↓ in lung cancer	SNAI1	Induction of the epithelial to mesenchymal transition, growth arrest, survival and cell migration	Kumarswamy et al., 2012
	miR-885-5p	↑ in liver cancer	CPEB2	Cell cycle progression	Siu-Chi Lam et al., 2017
	miR-9	↑ in ovarian cancer	E-CADHERIN	Calcium-dependent cell adhesion	Zhou et al., 2017
Reprogramming energy metabolism	miR-7	↓ in pancreatic cancer	LKB1	Cell metabolism, cell polarity, apoptosis and DNA damage response	Gu et al., 2017
	miR-1	↓in colorectal cancer	HIF1α	Activation of genes involved in metabolism,angiogenesis,erythropoiesis and glycolysis	Xu et al., 2017
	miR-150	↑ in glioma cells	VHL	Regulates the hypoxia inducible protein HIF in normoxic conditions	Li et al., 2017
Evading immune destruction	miR-146	↑ in colorectal cancer	IRAK1; TRAF6	Initiates innate immune response against foreign pathogens; activation of NFKB by TNFRSFs	Rusca and Monticelli, 2011; Khorrami et al., 2017
	miR-152	↓ in gastric cancer	B7-H1	Costimulatory signal, essential for T-cell proliferation and production of IL10 and IFNG	Wang Y. et al., 2017

affecting the tumor immune microenvironment (De Mattos-Arruda et al., 2015). It is also true that some microRNAs may have a dual role in different cancer types, acting as tumor suppressor or onco-miR. miR-181a when overexpressed, was described in human glioma cells to induce apoptosis and dampen cell invasion (Shi et al., 2008) and migration in non-small cell lung cancer (NSCLC) (Cao et al., 2017). Interestingly, in human gastric cancer cells, miR-181a has been reported to be an onco-miR, promoting cell proliferation, wound healing invasion and EMT targeting RASSF6 (Mi et al., 2017). Thus, the complexity of the involvement of microRNAs in cancer is high and disentangling the dense net of RNAs interaction in order to build a complete and clear scenario will be a real challenge.

MICRORNA AS CANCER BIOMARKERS

As we have mentioned, it has become evident that microRNAs are involved in many aspects of cancer and because of their mechanism of action they control a big network of targets rather than few specific genes. This means that profiling microRNAs may give insights on complex processes hidden in numerous target genes, helping researchers to find new useful biomarkers.

The definition of biomarker evolved with time and is not unique, but it could be summarized as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Strimbu and Tavel, 2010). As a matter of fact, microRNAs possess most of the characteristics of the ideal biomarker, considering analytical criteria and clinical utility. They are specific to the pathology of interest, a reliable indication of the disease before clinical symptoms appear and sensitive to physiological or pathological changes. First demonstrations of the ability of microRNA expression patterns to be classifiers came in the first decade of 2000. Lu and colleagues implemented a bead-based microRNA profiling method in order to assess microRNA expression in normal and tumor tissues. Unexpectedly, they observed that precise pattern of microRNAs expression can, not only distinguish tumor origin, but also the degree of differentiation and classify poorly undifferentiated tumor tissues (Lu et al., 2005). Other evidences came from microRNA signatures that could discriminate between lung tumor tissues and correspondent non-tumor tissues. Differential expression was also seen between adenocarcinoma (AD) and squamous cell carcinoma (SCC) tissues and between distinct prognosis (Yanaihara et al., 2006). A wider analysis on 22 different types of tumor tissue, revealed a signature of 48 microRNAs able to reach a classification accuracy >90% (Rosenfeld et al., 2008). It is important to mention how NGS (Next Generation Sequencing) technologies revolutionized this field becoming progressively fundamental tools for personalized medicine (Schweiger et al., 2011). Even in microRNA studies these methods revealed completely new information which probably would not have been unveiled with standard techniques. In view of the big amount of data coming from NGS, new biomarkers have been discovered starting with an agnostic discovery platform methodology (Leidner et al., 2012; Wu et al., 2012) allowing researchers to be unbiased on their findings. There are some limitations that come together with the power of NGS, like costs, time-consuming experiments and management of big amount of data.

Pattern of microRNAs expression may be used to classify sub-population of patients in order to choose the right strategy in the clinical practice. However, we have to be aware that the microRNA signatures as biomarkers are not always due to a direct biological mechanism, but also to indirect specific consequence of the disease. In the following paragraphs, we report some examples (**Table 2**).

Biomarkers for Cancer Diagnosis and Sub-Typing

Biomarkers can stratify patients upon different aims. One of the first clinical questions could be to understand whether the physician is facing a pathological condition. Therefore, discriminating between tumor tissues and non-tumor tissues is extremely important. The most recurrent example is miR-21 which is over-expressed in many cancer types (Iorio et al., 2005, 2007; Volinia et al., 2006; Markou et al., 2008; Hezova et al., 2015; Kapodistrias et al., 2016; Parafioriti et al., 2016; Calatayud et al., 2017; Chen et al., 2017; Cui et al., 2017; Nakka et al., 2017). The problem of using such microRNA as a biomarker is the absence of specificity. Therefore, signatures of a pattern of microRNAs are generally preferred to deliver a specific diagnosis. A nine microRNAs signature was able to discriminate between breast cancer tissues and normal cancer tissues collected by TCGA, with a high accuracy value and AUC of 0.995 (Xiong et al., 2017). Another example comes from the He group which found five microRNAs (miR-424, miR-326, miR-511, miR-125b-2 and miR-451) able to provide high diagnostic accuracy of hepatocellular carcinoma starting from microRNA expression profiles of 377 hepatocellular carcinoma patients (Lu et al., 2017). As finding the pathological condition is relevant, the step forward is to understand what type of condition the clinician is facing. It is well-known that each cancer type is composed of several subtypes coming from different cellular origins and each of them has to be treated accordingly. Thus, it is important to discriminate among them and several studies pointed at this aim. A study on muscle-invasive bladder cancer in 2016 revealed a signature of 63 microRNAs able to discriminate between basal and luminal tumors and a 15 microRNAs based signature able to show basal and luminal tumors with apparent fibroblast infiltration (Ochoa et al., 2016). Similarly, Blenkiron and colleagues performed a model-based discriminant analysis for basal-like and luminal A breast tumors finding a set of microRNAs able to discriminate between those groups (Blenkiron et al., 2007). Another approach by the Jang lab exploited the expression of 1,733 microRNAs to build an unsupervised clustering in order to distinguish subtypes of pancreatic tumors. As result, they found 3 subtypes which could be associated with patient prognosis (Namkung et al., 2016). In lung cancer, in the data of the Volante lab, 10 microRNAs were able to distinguish between lung neuroendocrine (NE) tumors histotypes, 9 of which also discriminated between carcinoids and high-grade NE carcinomas (Rapa et al., 2015). In addition, combination of miR-21 and miR-205 was found to be able to distinguish lung AD from SCC (Lebanony et al., 2009) and this can be further improved with the analysis of miR-375 (Patnaik et al., 2015). As a matter of fact, we demonstrated the non-perfect reliability of miR-205 in discriminating AD vs. SCC lung cancer histotypes (Del Vescovo et al., 2011).

Biomarkers for Cancer Progression

Understanding the aggressiveness and progression of cancer via prognosis of the patient is of enormous relevance in clinical practice. microRNAs are able to predict patient prognosis in several types of cancer. Here show some examples from lung cancer. Let7 was found to be down-regulated in lung cancer *in vitro* and *in vivo*. A cohort of 143 lung cancer tissues was analyzed for the expression of let7 which resulted significantly down-regulated compared to normal tissues. Moreover, reduced let7 associates with higher disease stages and poor post-surgery survival and prognosis. Taking into account only the AD samples, these distinctions are maintained (Takamizawa et al., 2004). A wider analysis led to discover a 5 microRNAs signature (miR-221 and let7a protective, while miR-137, miR-372 and miR-182-3p risky) able to discriminate between NSCLC patients with higher

TABLE 2 | (part 1) Examples of microRNAs as biomarkers.

Type of classification	microRNA	Type of cancer	Reference
Cancer VS Healthy	↑ miR-21	Breast cancer; lung cancer; stomach cancer; prostate cancer; colon cancer; pancreatic cancer; ovarian cancer; esophagus cancer; Ewing's sarcoma; liposarcoma; Wilm's tumor; osteosarcoma; oral tongue cancer	Iorio et al., 2005, 2007; Volinia et al., 2006; Markou et al., 2008; Hezova et al., 2015; Kapodistrias et al., 2016; Parafioriti et al., 2016; Calatayud et al., 2017; Chen et al., 2017; Cui et al., 2017; Nakka et al., 2017
	↑ miR-21, miR-96, miR-183, miR-182, miR-141, miR-200a, miR-429; ↓ miR-139 and miR-145	Breast cancer	Xiong et al., 2017
	↓ miR-424, miR-326, miR-511, miR-125b-2, miR-451	Hepatocellular cancer	Lu et al., 2017
Sub-typing	Panel of 63 microRNAs	Basal and luminal muscle-invasive bladder cancer	Ochoa et al., 2016
	Panel of 137 microRNAs	Basal and luminal A breast cancer	Blenkiron et al., 2007
	Panel of 19 microRNAs	Pancreatic ductal adenocarcinoma	Namkung et al., 2016
	miR-15a, miR-22, miR-141, miR-497, miR-129-5p, miR-185, miR-409-3p, miR-409-5p and miR-431-5p, miR-129	Lung neuroendocrine cancer histotypes	Rapa et al., 2015
	miR-21, miR-205, miR-375	Lung adenocarcinoma and squamous cell carcinoma	Lebanony et al., 2009; Del Vescovo et al., 2011; Patnaik et al., 2015
Cancer progression	↓ let7	Lung cancer	Takamizawa et al., 2004
	miR-221 and let7a protective, while miR-372 and miR-182-3p risky	Lung cancer	Yu et al., 2008
	Panel of 20 microRNAs	Lung cancer	Yanaihara et al., 2006
	↓ miR-448	Lung cancer	Shan et al., 2017
	↓ miR-383	Lung cancer	Shang et al., 2016
	↑ miR-187	Lung cancer	Peng et al., 2016
	↓ miR-187	Renal cell carcinoma	Zhao et al., 2013
	↓ miR-187	Ovarian cancer	Chao et al., 2012
Cancer Therapy	↑ miR-21	Colon cancer (poor fluorouracil based adjuvant chemotherapy outcome)	Schetter et al., 2008, 2009
	↑ miR-21	Pancreatic cancer (poor fluorouracil-based adjuvant chemotherapy outcome)	Hwang et al., 2010
	↑ miR-21	Lung cancer (poor platinum-based chemotherapy outcome)	Gao et al., 2012
	↑ miR-448	Lung cancer (cisplatin resistance)	Fang et al., 2016, 2017
	↓ miR-138	Lung cancer (cisplatin resistance)	Wang et al., 2011
	↓ miR-10b	Pancreatic cancer (highly predictive response to gemtabicine-based multimodality neoadjuvant therapy)	Preis et al., 2011
	↓ miR-148	Colorectal cancer (poor fluorouracil and oxaliplatin-based therapy outcome)	Takahashi et al., 2012
	miR-221, miR-222, miR-331, miR-451, miR-28, miR-151, miR-148a, miR-93, miR-491	Diffuse large B-cell lymphoma (prediction of OS and PFS after rituximab and chemotherapy treatment)	Montes-Moreno et al., 2011
	↑ miR-31-3p ↓ miR-592	Colorectal cancer (poor anti-EGFRmAb therapy outcome)	Mosakhani et al., 2012
	↑ signature of let7c, miR-99a, miR-125b	Colorectal cancer (good cetuximab and panitumumab outcome)	Cappuzzo et al., 2014
	↑ miR-31-3p, miR-31-5p	Colorectal cancer (lower PFS after anti-EGFRmAb therapy)	Igarashi et al., 2015; Mlcochova et al., 2015
	↑ miR-200c	Lung cancer (good of EGFR-TKIs therapy outcome)	Li et al., 2014b
	A panel of 29 microRNAs	Renal cell carcinoma (TKIs therapy outcome)	Garcìa-Donas et al., 2016

(Continued)

TABLE 2 | Continued

miR-181a-5p, miR-339-5p	Hepatocellular carcinoma (prediction of sorafenib therapy outcome)	Nishida et al., 2017
↑ miR-183	Renal cancer (less efficacious cancer cytotoxicity by natural killer cells)	Zhang et al., 2015
↑ miR-6826, miR-6875	Colorectal cancer (poor vaccine therapy outcome)	Kijima et al., 2016

From diagnosis to the choice of therapeutic intervention.

or lower median overall survival (OS) independently from stage or histology. However, this signature is able to predict patient survival within histological type AD or SCC (Yu et al., 2008). With a similar strategy, a pattern of unique 15 microRNAs was able to discriminate between lung SCC and normal tissues, while a signature of 20 microRNAs was able to predict the OS (Raponi et al., 2009). Some of these microRNAs were more significant, like miR-146b which had the highest prediction score within 3 years, and some had already been linked to lung cancer in other studies like let-7 and miR-155 (Yanaihara et al., 2006). Interestingly, in all these studies, the different isoforms of let-7 found, were down-regulated in patients with poor prognosis. More recent data show that low expression of miR-448 associates with lung SCC progression and poor patients overall survival (Shan et al., 2017). Reduced expression of miR-383 was found in NSCLC tumor tissues compared to adjacent non-tumorous samples and moreover, low miR-383 expression associated with poor post-operative prognosis (Shang et al., 2016). miR-448 and miR-383 are down-regulated, acting like tumor-suppressors, also in ovarian cancer (Lv et al., 2015), hepatocellular carcinoma (Zhu et al., 2015; Chen et al., 2016), colorectal cancer (Li et al., 2016), breast cancer (Li et al., 2011), Hodgkin lymphoma (Paydas et al., 2016), glioma (He Z. et al., 2013; Xu et al., 2014) testicular carcinoma (Lian et al., 2010; Huang et al., 2014) and medulloblastoma (Li et al., 2013). Another study revealed that miR-187 expression was significantly increased in NSCLC tissue samples compared to adjacent non-lung tumor tissues and that this condition associated with TNM classification and shorter OS (Peng et al., 2016). Interestingly, miR-187 has been found down-regulated in clear cell renal cell carcinoma cells (Zhao et al., 2013) but up-regulated in ovarian cancer cells (Chao et al., 2012). However, in both cases the studies agree with what occurs in lung cancer, where low miR-187 level of expression is associated with poor patient survival.

Biomarkers for Cancer Therapy

As a consequence of the intricacy of underlying driving mechanisms of cancer, therapeutic efficacy of a single treatment can change depending on the patient and its type of cancer. microRNAs have been associated to, and also predictive of, therapeutic outcome. Here we report cases of some of the main standard cancer treatments.

miR-21 seems to be a general signal for chemotherapy resistance. In 2008, Schetter and colleagues found that miR-21 expression, in typical colon AD from patients treated with fluorouracil based adjuvant chemotherapy, is higher in patients with a poor therapy outcome (Schetter et al., 2008, 2009). Similar results were obtained for pancreatic cancer (Hwang et al., 2010). Even in lung cancer, high-expression of miR-21 was associated with chemotherapy resistance in tissues of patients who had undergone platinum-based chemotherapy treatment (Gao et al., 2012). It was shown that A549/DDP lung AD cell line has a lower expression of eIF3a compared to its parental cell line, and it displays chemoresistance to cisplatin. miR-488 targets the 3′UTR of eIF3a transcript enhancing sensitivity to the treatment and inhibiting cell proliferation, migration and invasion (Fang et al., 2016, 2017). Another study reported an increased sensitivity of A549/DDP cells to cisplatin after up-regulation of miR-138. The excision repair cross-complementation group 1 (ERCC1) was targeted by miR-138 and the result was the down-regulation of the protein correlating with increased levels of miR-138 in A549/DDP cells (Wang et al., 2011). In another study on pancreatic ductal AD, patients with resectable or locally advanced disease showed relative low miR-10b expression associated with highly predictive response to gemtabicine based multimodality neoadjuvant chemoradiotherapy. Moreover, by logistic regression, low miR-10b expression was able to predict surgery efficacy. miR-10b levels demonstrated significant ability in survival prediction (Preis et al., 2011). In CRC, miR-148 expression had a potential for predicting therapeutic efficacy of 5-fluorouracil and oxaliplatin in patients with stage IV colorectal cancer, as low levels of this microRNA associated with bad therapeutic response (Takahashi et al., 2012). Sensitivity to cisplatin treatment is, at least partially, regulated by miR-488 which targets eIF3a.

Besides chemotherapy, targeted therapy is an important standard of care for several tumors. Even in this field, microRNAs may be helpful. For diffuse large B-cell lymphoma (DLBCL), combination between chemotherapy and immunotherapy with rituximab has become a standard treatment. A 9 microRNAs signature was able to predict both OS and progression free survival (PFS) in DLBCL patients (Montes-Moreno et al., 2011). In a cohort of metastatic colorectal cancer patients wild type for KRAS and BRAF, a miR-31-3p up-regulation and miR-592 down-regulation were found associated with poor response to anti-EGFRmAb (Mosakhani et al., 2012). An Italian study reported a signature of three microRNAs (miR-let7c, miR-99a, and miR125b) able to predict EGFR monoclonal antibody therapy outcome in colorectal cancer patients. Indeed, high-level of signature expression showed a good discrimination capacity for patients which were more responsive to cetuximab or panitumumab compared to low responsive patients (Cappuzzo et al., 2014). In two independent studies, miR-31 was found to be associated with PFS after administration of anti-EGFRmAb in metastatic colorectal cancer patients. Mlchocova and colleagues

found both miR-31-5p and−3p, while Shinomura group only miR-31-5p, to be higher in patients with lower PFS compared to those with low levels of the microRNA (Igarashi et al., 2015; Mlcochova et al., 2015). microRNAs have been discovered to be predictive also of kinase inhibitors efficacy in hepatocellular carcinoma, renal cell carcinoma and NSCLC (Li et al., 2014b; Garcìa-Donas et al., 2016; Nishida et al., 2017).

An emerging field in cancer treatment is immunotherapy. Some studies describe microRNAs as biomarkers of immunotherapy efficacy. In a report on 82 renal cancer patients and 19 healthy individuals, miR-183 has been found up-regulated in sera associated to less efficacious cancer cytotoxicity by natural killer cells, which are the effectors of the IL-2 immunotherapy (Zhang et al., 2015). Nagano group described that miR-6826 and miR-6875 can be good predictor of vaccine treatment efficacy in metastatic CRC, where high expression in plasma of two microRNAs was associated with poorer prognosis (Kijima et al., 2016).

CIRCULATING MICRORNAS

Over the last two decades, it has been demonstrated that a substantial number of microRNAs are present outside cells in blood and other body fluids, the so-called "circulating microRNAs" (c-microRNAs). C-microRNAs have been reported to be very stable under harsh conditions and able to survive high temperatures, extreme pH, and RNase activity. As reviewed by Makarova and colleagues (Makarova et al., 2016) they are often found in association with small membranous particles (extracellular vesicles) and mostly with RNA-binding proteins (Ago2, HDL, etc.). The extracellular vesicles (EV) are represented by a various population of membranous particles with different origins and sizes. Microvesicles originate through the budding of the plasma membrane and have a size around 100–1,000 nm. Exosomes, around 40–100 nm in size, are generated after the fusion of multivesicular bodies with the plasma membrane. The presence of these different carrier options leads researchers to think of selective microRNA sorting and secretion processes, not excluding stochastic (non-selective) ones. Moreover, the pool composition of the microRNAs is different intra- and extracellularly. Unfortunately, the exact mechanisms underlying these processes have to be discovered yet. Even though extracellular vesicles biogenesis is varied, only ceramide-dependent mechanism has been reported as one of the responsible for microRNA secretion so far (Kosaka et al., 2010). About sorting, it has been suggested that the affinity between the RNA and the raft-like membrane regions of the multivesicular bodies (MVBs) can guide it (Janas et al., 2015). In another study, Squadrito et al. (2014) reported that sorting of microRNAs to exosomes is partially regulated by the changes in expression of the targets inside the cell. The finding of a different microRNA sorting in exosomes depending on the KRAS status (Cha et al., 2015), adds concrete value on the selective sorting hypothesis. In 2013, Sànchez-Madrid group (Villarroya-Beltri et al., 2013) performed several microarrays analyses of activation-induced changes in the microRNA and mRNA profiles among T-lymphoblasts and their exosomes. They obtained a discordance between intracellular and extracellular microRNA and mRNA pool composition, demonstrating once again that the sorting into exosomes is not—at least completely—passive. Interestingly, they reported a short sequence motif (GGAG) enriched in exosomal microRNAs. Among the many heterogeneous nuclear ribonucleoproteins (hnRNPs) that precipitated with intracellular and exosomal microRNAs, only hnRNPA1 and hnRNP2B1 seemed to bind exclusively the latter. Another study demonstrated that Vps4A, a key regulator of exosomes biogenesis, seemed to regulate the sorting of oncogenic and oncosuppressive microRNAs in exosomes, favoring the inclusion of the first ones (Wei et al., 2015). Furthermore, it has been described how, in B cells, 3′end adenylated microRNAs appear to be enriched in cells compared to 3′ end uridylated isoforms which are more present in exosomes (Koppers-Lalic et al., 2014). What remains really unclear is the mechanism of sorting, if present, and of secretion of AGO-microRNA complexes. To date, the study reported by Turchinovich and colleagues, leads to think that the majority of these complexes is freed in a non-selective manner, because of the positive correlation between the content of c-microRNA in culture media and the increase of cell death (Turchinovich et al., 2011). Moreover, the Cayota group described via RNA-seq analysis, how expression values of individual microRNAs in intracellular fractions of MCF-7 cells after a certain threshold, correlated directly with extracellular values, suggesting a passing mechanism of release also for extracellular vesicles related microRNAs (Tosar et al., 2015). However, these conclusions do not wipe out at all the possibilities of a parallel selective secretion.

The presence of putative precise processes underlying c-microRNAs suggests that they could have an intriguing role in cell-cell communication. For instance, it was demonstrated that microRNAs enriched in extracellular vesicles derived from bone marrow mesenchymal stem cells can be absorbed by tubular epithelial cells resulting in the inhibition of expression of the known targets (Collino et al., 2010). Furthermore, as it has been reviewed (Neviani and Fabbri, 2015), c-microRNAs can influence cancer cells and their surrounding environment both targeting mRNAs and functioning as receptor-like systems. Much of the data supporting this way of cell-cell communication is done through *in vitro* systems, pushing for new validating studies *in vivo* which may confirm this hypothesis. One of the unclear point which can be argued is whether the actual amount of c-microRNAs is enough to drive expression changes in recipient cells *in vivo*. Some studies report that the average amount of microRNAs in exosomes is about 1 unit per exosome (Chevillet et al., 2014; Guzman et al., 2015). This very low amount may lead to some skepticism around the role of c-microRNAs in cell-cell communication. However, extracellular vesicles associated microRNAs are a small percentage of the total pool of c-microRNAs (Arroyo et al., 2011) and in addition, this semi-quantitative reasoning is far too simplistic, not taking into account, for example, the accumulation of microRNAs in recipient cells and, being a median measure, doesn't consider the content heterogeneity of extracellular vesicles.

Not only microRNAs from tissue can be used to create pattern of signatures able to classify group of patients but also c-microRNAs from liquid biopsies are becoming an increasing source of information (Chen et al., 2008). c-microRNAs as biomarkers have some advantages like great stability, resistance to ribonucleases and to severe physicochemical conditions in body fluids, increasing the feasibility of their use in clinical applications (Mitchell et al., 2008). Another important aspect is the compliance of the patients. Indeed, c-microRNAs are extracted from several body fluids coming from liquid biopsies, which are much less invasive and painful for the patients compared to the standard methods. Moreover, the cost and time for the processing is lower than non-liquid samples, promising big step toward the implementation of personalized medicine. Thus, the interest of the scientific community has grown intensively as demonstrated by the number of articles published in recent years at the entry "circulating microRNAs cancer biomarker" on PubMed (2012 ➔ 86; 2013 ➔ 148; 2014 ➔ 188; 2015 ➔ 239; 2016 ➔ 201). Moreover, there are some reviews which try to collect as much as possible the huge amount of information on circulating microRNAs as cancer biomarkers (Del Vescovo and Denti, 2015; Armand-Labit and Pradines, 2017; Matsuzaki and Ochiya, 2017; Zhao et al., 2017).

MIRSNP

microRNAs exert their function through an interaction with seed sequence on either 3′UTR, 5′UTR or the coding sequence of a target mRNA. The hybridization between the two RNAs follows the Watson-Crick base pairing rules and thus it is guided by the formation of a stabilized double strand structure. Thus, when even single nucleotide changes occur in the sequence of either of the two interactors (miRSNP), the stability of the contact is affected and so is the functional outcome. Indeed, single nucleotide polymorphisms (SNPs) can affect microRNA expression and function and they can be present in the sequence of the microRNA or on its target genes (**Figure 2**). SNPs may be present also in the sequence of genes involved in the biogenesis of microRNAs, thus affecting their level of expression. These changes can influence the above-mentioned patterns of microRNAs, creating completely new classes of patients based on association to risk of cancer or prediction to therapy. Moreover, as reviewed by Del Favero group, it should be considered that SNP density is higher in the flanking region of the microRNA sequence compared to microRNA genes themselves and mature form of microRNAs has lower SNP density than the pre-microRNA. Interestingly, the seed sequence has the lowest SNP density, highlighting their evolutionary and functional importance (Cammaerts et al., 2015).

In literature, there are numerous reports describing cancer risk association with SNPs related to microRNAs life. In a meta-analysis by Liu and colleagues (Liu H. et al., 2017), conducted on ten studies with 6,000 cases and 7,664 controls, a significant association of miR-608 rs4919510 polymorphism with decreased cancer risk via recessive model (CC vs. GG + GC) was found. Interestingly, the same polymorphism has been already described to predict the clinical outcome in patients with different cancer types (Lin et al., 2012; Zheng et al., 2013; Pardini et al., 2015). As mentioned above, SNPs associated with microRNAs can alter several of their usual processes. One of the most common alteration is the degree of target suppression. rs73239138 polymorphism in miR-1269 was associated with increased susceptibility to hepatocellular carcinoma (HCC) and HBV-related HCC in a positive dominant model where genotypes with the A allele increased the risk to cancer (Min et al., 2017). It was shown that in HCC cell lines the over-expression of the miR-1269 variant led to a decreased inhibition of cell growth compared to the over-expression of the wild type microRNA. The authors confirmed the biological outcome demonstrating that the polymorphism on miR-1269 produced a dampened suppression of pErK1/2, SPATS2L and LRP6 compared to the wild type variant, where the last two genes showed to have a 3′UTR binding site for miR-1269. In a study on an Indian population (Sibin et al., 2017), miR-196a2 expression varied with age, tumor grade and tumor type among glioma patients' tissues but not with different genotype of the microRNA. However, they found a significant difference in the expression of its mRNA target HOXC8 depending on different genotypes where CC and TT showed decreased and increased expression, respectively. Remarkably, out of 72 sample pairs of tumor tissues and blood samples, 19,44% showed different genotype of miR-196a2 in the tissue compared to the blood suggesting a critical role in tumorigenesis and in changing of tumor grade. Other evidences pointed out the importance of polymorphisms altering the ability of the microRNAs to post-transcriptionally inhibit gene expression of their target in colorectal (Liu Y. et al., 2016) and gastric cancer (Liu C. et al., 2016). Along with a negative effect of a SNP on microRNA sequence, this variation may lead also to positive effects, increasing binding capacity of the microRNA to its target (Gong et al., 2012). To the best of our knowledge, there are no reports of this scenario in cancer yet, neither *in vitro* nor *in vivo*.

Undoubtedly, variations in the sequence of microRNAs can also affect their maturation. Bioinformatic analysis of Gibbs free energy on the structure of the miR-146a stem loop showed that the G allele of the rs2910164 polymorphism increased the stability factor of the overall structure, suggesting that, the association between the C carrier allele in the Iranian population under study may correlate with lower expression of miR-146a and thus higher incidence of the presence of its target Her2 in breast cancer (Meshkat et al., 2016). Indeed, miR-146 is significantly higher in triple-negative breast cancer compared to non-triple-negative breast cancer (Garcia et al., 2011). The already mentioned polymorphism on miR-196a2 was associated in other cancer studies to the microRNA maturation, changing the miR-196a2 expression (Hu et al., 2008, Hoffman et al., 2009). Another common scenario is the presence of a polymorphism in the microRNA binding site of an mRNA target. In a study on 325 colorectal cancer (CRC) patients and 977 normal individuals, the polymorphism rs7930 in the 3′UTR of TOMM20 was found to be associated with CRC susceptibility and the G allele described as the risk allele. Via *in silico* target analyses, miR-4273-5p was

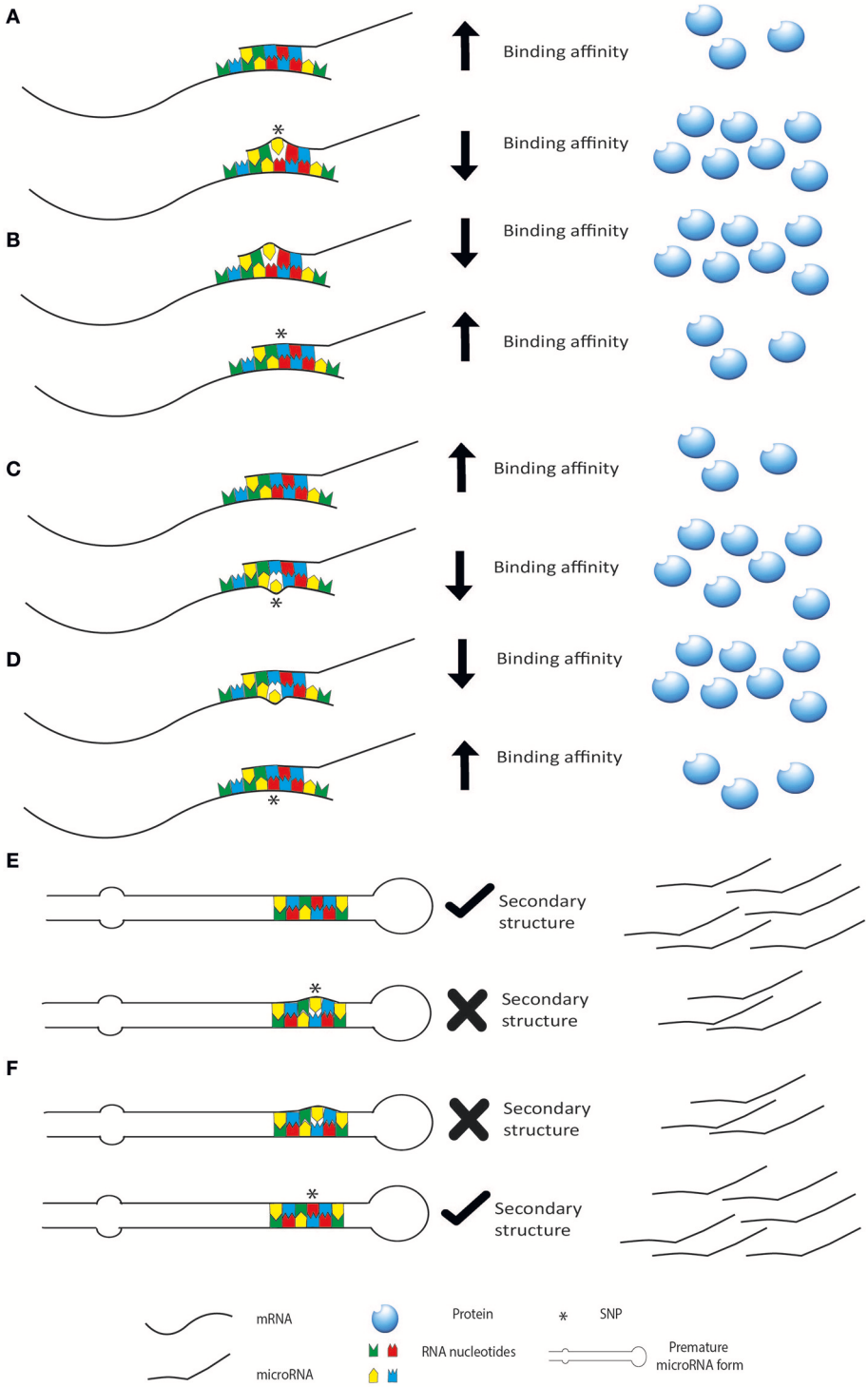

FIGURE 2 | miRSNP can affect microRNA biogenesis and activity. SNPs may be present on the microRNA decreasing **(A)** or increasing **(B)** its binding affinity for the target mRNA. SNPs may be present in the binding site of a target mRNA decreasing **(C)** or increasing **(D)** binding affinity (or creating new binding sites). In this last scenario are represented also SNPs in genes of the microRNA biogenesis machinery. These SNPs usually affect the regulation of the genes increasing or decreasing binding affinity of post-transcriptional regulators like microRNAs. SNPs may also affect the secondary structure of premature forms of the microRNAs decreasing **(E)** or increasing **(F)** their maturation.

predicted to interact with rs7930. Validation by luciferase assay in human CRC cell lines demonstrated that the G allele plasmid did not have any effect on the reporter compared to a stronger effect of the A allele plasmid. Moreover, cell lines with the AA genotype showed a considerably stronger dampen in TOMM20 levels than those with the AG genotype (Lee et al., 2016). On two stages study made of 2347 cases and 3390 controls in total, Ke and colleagues found that the polymorphism rs1062044 on the sequence of LAMC1 produces an increased risk of colorectal cancer in the GA genotype compared to GG. Moreover, it decreases the ability miR-423-5p to bind LAMC1 in CRC cancer cell lines (Ke et al., 2017). The presence of a polymorphism on the binding site could also lead to a positive effect, thereby creating a new illegitimate binding site. Bartel group found that polymorphism SNP34091 in the 3'-UTR of MDM4 creates a new binding site for miR-191 in ovarian cancer (Wynendaele et al., 2010).

Polymorphisms on genes involved in the microRNA biogenesis can also have an impact on cancer progression. Mullany and colleagues, through RNA-seq and GWAS analysis of colon cancer tissues, found 24 microRNAs which were deregulated in the presence of SNPs significantly associated with altered mRNA expression or cancer risk. In particular, rs2740349 (GEMIN4) and rs235768 (BMP2) were shown to be associated with microRNA expression variation, with up-regulation corresponding to the variant genotypes (Mullany et al., 2016). Interestingly, this up-regulation is associated with a down-regulation of the mRNAs of biogenesis genes, implicating new roles for these genes or other mechanisms of microRNA expression influence. In addition, Rotunno and colleagues found that RNASEN/rs640831, present in the GTACCT haplotype was associated with variation in expression of 56 microRNAs, both up- and down-regulated (Rotunno et al., 2010). One mechanism of action of these SNPs is the change in binding affinity of a regulator of the transcript like a microRNA. Jiang and colleagues genotyped 24 SNPs in a cohort of 878 breast cancer patients and 900 controls. They found that polymorphism rs417309 is associated with higher breast cancer risk (Jiang et al., 2013). Moreover, this SNP is placed on the 3′UTR of the DGCR8 mRNA, affecting the binding ability of miR-106b and miR-579. However, the imperfect relationship between up-/down-regulation of microRNA biogenesis-related genes and up-/down-regulation of microRNAs, suggests a series of effects which are far to be completely clear and understood.

Behavioral changes of microRNAs upon SNPs can also affect the performance of cancer drugs. Pharmacogenomics studies how single genome or transcriptome variations can affect pharmacokinetics (PK) and dynamics (PD). A lot of attention has been drawn to microRNAs as possible players in this area. The contribution of microRNAs to PK and PD has been studied also via bioinformatic tools. Some genes are specific to PK, some to PD, whereas others to both. *In silico* data hint to a higher relevance of post-transcriptional regulation by microRNAs on PD unique genes compared to PK unique genes (Rukov et al., 2011). As a matter of fact, the latter show on average a shorter 3′UTR with a less dense presence of predicted target binding site for microRNAs, compared to the former. Among some genes coding for drug metabolizing enzymes (DMEs), transporters and nuclear receptors, CYP1A2, CYP2B6, CYP2D6, CYP3A4, NR1I2, and UGT2B7 were sequenced for their 3′UTR in a population of 30 South Africans (Swart and Dandara, 2014). 40 out of 52 SNPs detected were predicted to potentially create or abolish microRNA binding sites, thus affecting regulation capacity and expression of those genes. Despite the low number of patients enrolled, this study highlights once more the engagement of microRNAs in pharmacogenomics. Therefore, an increasing interest in creating web tools to analyze miRSNP and drugs has grown.

Mir2Drug is a database able to calculate the influence of miRSNP in drug efficiency. It considers the sequence 30 bp up- and downstream a known SNP in the 3′UTR of target genes, and calculates all the predicted binding sites for microRNAs in that region, analyzing the change in free energy from wild type to variant genotype. Upon significant differences, Mir2Drug associates these SNPs as either direct or indirect drug targets. Therefore, it provides comprehensive annotation information on miRSNP belonging to drug target genes (Wang X. et al., 2017).

SMiR-NBI is another bioinformatic tool available on the web, which provides insights on possible pharmacogenomic biomarkers characterized by microRNAs, comprehending a network connecting small molecules to microRNAs regulation (Li et al., 2014a). This growing interest on miRSNP and pharmacogenomics comes from several works and here we try to review examples from different cancer types. A standard of care therapy for advanced lung cancer patients is the platinum-based chemotherapy. This kind of therapy leads to a spectrum of toxicities with different degree of severity. A study of Fang et al. (2016) found that the polymorphism rs2042553 of miR-5197 significantly associates with severe toxicity after platinum-based treatment. Moreover, miR-605 polymorphism rs2043556 was associated with hepatotoxicity, while miR-27a rs895819 was related to gastrointestinal toxicity. Platinum-based therapies are often combined with other drugs like gemcitabine or paclitaxel. Geng et al. (2016) studied the effect of different regimen of chemotherapy based on cisplatin plus paclitaxel, gemcitabine or Changchun vinorelbine, in a cohort of advanced NSCLC patients. They found that polymorphism rs11077 in XPO5, a transport factor involved in the export of pre-microRNAs from the nucleus to the cytoplasm, is associated in AA genotype to a worse prognosis in a chemotherapy regimen compared to the AC genotype. Human Pregnane X Receptor (PXR) induces expression of DMEs, thus it can potentially influence the efficacy of several anticancer drugs. In a study on 96 Indian breast cancer patients (Revathidevi et al., 2016), genomic DNA from blood samples was sequenced for PXR 3′UTR. Among 12 SNPs already reported in several databases, 5 SNPs were observed and in particular, for SNPs rs3732360 and rs3732359 the proportion of the mutant allele is higher compared to the wild type in the studied population. These two polymorphisms conferred a new binding site for miR-500a-3p and decreased the binding of miR-532-3p which is known to play a role in doxorubicin cardiotoxicity (Wang J.-X. et al., 2015). In fact, the observed SNPs either created new binding sites for microRNAs, or abolished some of them, or strengthen or dampened the binding capacity of others.

Therefore, overall regulation of PXR could be affected impacting on the metabolism of drugs. As a matter of fact, microRNA predicted to be influenced by these SNPs are also involved in treatment efficacy and doxorubicin cardiotoxicity, as pathway analysis revealed. Even in non-solid tumors microRNA variation may impact on treatment efficacy and toxicity. Lòpez-Lòpez and colleagues studied possible associations between miRSNP and adverse reactions after methotrexate administration in Acute Lymphoblastic Leukemia (ALL) (Lopez-Lopez et al., 2014). They showed that SNP rs639174 in DROSHA is associated with vomit during consolidation of methotrexate treatment, as well as rs56103835 in pre-miR-453. Moreover, rs12894467 in pre-miR-300 is associated with hepatic toxicity and hyperbilirubinemia in induction. However, the same group found that none of the miRSNP genotyped in a Spanish population of 152 ALL affected children, is associated with Vincristine-related neurotoxicity (Lopez-Lopez et al., 2016), highlighting how microRNAs are not involved randomly in every process, but they are selectively and directly responsible or indirectly involved in some divergent mechanism.

It is worth mentioning that also big sequence changes like INDELs in microRNAs related genomic regions can have an impact on how drugs are affected by human body and vice versa. Garcìa-Ortì et al. (2012) found 19 microRNAs associated with gene copy number variations in genomic regions where they are located, in acute myeloid leukemia (AML) cells. 4 out of 19 had NF1 as a potential target gene but only miR-370 was then validated. Patients analysis showed that NF1 down-regulation by either miR-370 over-expression or NF1 gene deletion is common in AML. Thus, considering that NF1 deficiency leads to RAS activation, patients with over-expression of miR-370 may potentially take advantage from RAS or mTOR inhibitors (Parkin et al., 2010). Another study (Bruhn et al., 2016), pinpointed that different lengths in the 3′UTR ATP binding cassette (ABC) membrane transporter P-gp (ABCB1) may alter the presence of several microRNA binding sites. Actually, imatinib resistant leukemia cell lines expressed shorter 3′UTR potentially losing some regulating sequences. Indeed, the shortening of ABCG2 (another ABC transporter) 3′UTR removes miR-519c binding site, therefore contributing to drug resistance (To et al., 2009).

LIMITATIONS OF MICRORNAS AS TOOLS FOR PERSONALIZED MEDICINE

microRNAs are intensively studied as tools for personalized medicine because they encompass many ideal characteristics for fast and robust analysis, which is needed in clinical practice. As a matter of fact, they are generally stable due to protein based carriers or EVs engulfment. Moreover, the detection is easier so far, considering the low amount required, the hybridization methods criteria which avoids the production of complex probes like antibodies for proteins and the accessibility of the technologies. The ability of microRNAs to fine tune the gene expression enables these markers to be more sensitive in the pathology follow-up. On the contrary, biomarkers like ctDNA (circulating tumor DNA), which is a promising new biomarker for cancer practice, being strictly linked to genomic mutation analysis, suffer from uncertainty in ongoing follow-up (Nadal et al., 2017). However, some problems still limit the use of microRNAs in personalized medicine. The source of microRNAs has to be managed accordingly and the influence on recovery and final outcome may be substantial, especially for RNA extracted from biofluids. Regarding the detection, the short sequence of microRNAs impedes an easy design of probes, limiting also the discrimination between pri-, pre- and mature forms. Moreover, despite the ease of use and accessibility of qRT-PCR, ddPCR, microarrays and NGS as main detection techniques for nucleic acids, they lack strong sensibility and accuracy, especially at single base resolution. Another key point is the normalization of the signal. In fact, as the analysis is about a relative expression, the choice of a good normalizer is fundamental and challenging (Masè et al., 2017). In addition to these analytical problems, the complex biology of microRNAs increases the obstacles toward a full comprehension of these small non-coding RNAs. As a matter of fact, functional studies with microRNAs suffer from absence of physiological conditions, thus when over-expression studies are performed it should be taken into account that microRNAs generally act with low quantities and more than one on a single target.

Indeed, personalized medicine is going through the use of c-microRNAs instead of tissue-derivatives. Despite the clear high potential of c-microRNAs in the future personalized medicine, technical difficulties to perform robust and comparable profiling of these small nucleic acids have impeded progress to develop an approved clinical diagnostic assay (Jarry et al., 2014).

The problems come through three different steps in the analysis of c-microRNAs: pre-analytical, analytical and post-analytical phase. Therefore, from where and how c-microRNAs are extracted, how we detect them and how we process the data, still leads to great variability among different studies. Pre-analytical variables are those factors which can affect the composition of the sample to be tested: from patient's conditions variability to sample handling. Firstly, it has to be considered that usually, c-microRNAs are in low titer in biofluids compared to microRNAs in sample tissues. Considering the study of Tewari and colleagues, the concentration of microRNAs in plasma can be counted as from 100 to 9,000 copies per uL or, as shown in another study with ddPCR, even up to 23,000 copies/uL (Miotto et al., 2014). Similar results were found for cardiac injury induced microRNAs (Thompson et al., 2016). Moreover, the lack of knowledge about the secretion and sorting of the microRNAs outside the cells, puts some limits on the patient's condition which would ensure reproducibility on the assays. Another challenge is represented by the contaminant microRNAs. It is known that c-microRNAs come from different cellular sources. Tewari group showed that blood cells are the major contributor to c-microRNAs, therefore variations in blood cells counts and hemolysis can affect the interpretation of c-microRNAs signatures. They studied several oncological biomarkers reported in literature: many of them are highly expressed in blood cells. They demonstrated that this kind of c-microRNAs correlates with blood cell counts and that miR-122, which is not expressed in blood cells, doesn't follow this trend. Moreover, in hemolyzed

plasma samples, red blood cell-associated microRNAs vary up to 30-fold compared to non-hemolyzed samples, further proving that c-microRNAs pool is affected by blood cells composition (Pritchard et al., 2012). For these reasons, sample handling and processing become extremely important. Duttagupta and colleagues tried to discriminate between whole blood microRNAs derived from blood cells—"contaminant microRNAs"—and what they called "truly circulating microRNAs". Starting from whole blood samples and collecting different fractions from multiple centrifugation steps (**Figure 3**) they found that from fraction CS and S1 the content of "contaminant microRNAs" dropped, while the true c-microRNAs content stays more or less unchanged. On top of that, they showed that the variability of expression of marker c-microRNAs among a cohort of males and females decreases after the removal of the cellular contaminants originated from cellular microRNA signatures (Duttagupta et al., 2011). This points out how much the processing of the samples may affect the pool of c-microRNAs. Another study (Cheng et al., 2013) confirmed this variability, reporting that different plasma processing led, for the majority of c-microRNAs, to a variation in their expression levels, mainly due to different platelets and microvesicles content.

From the analytical point of view, it has to be considered that different extraction kits have distinct efficiencies in small RNAs recovery (Monleau et al., 2014). There are several challenges which involve also the detection and quantification of c-microRNAs. The design of qRT-PCR probes and assays is difficult because of (a) the shortness of microRNAs, (b) their wide range of concentrations, (c) the presence of many precursors and (d) the similarity in sequences. On the post-analytical side, we should mention that most of the detection methods rely on relative quantifications, therefore, an endogenous control is requested. This normalization analysis is needed in order to take into account the biological and technical inter-assay variability. So far, such control for c-microRNAs expression normalization, to be used for every tissue type, treatment and disease stage, has to be discovered yet. The most used in literature are miR-16, snRNA U6 and spiked-in cel-miR-39, but there is no general consensus from the scientific community and a different endogenous control is generally used for different purposes. For instance, among the several transcripts of U6, U6-1 was found to be have high variability and U6-2 was not detectable, in a study on sera from Hepatitis B infected patients and matched controls (Zhu et al., 2012). In two different plasma studies on CRC, miR-16 was found to have quite high stability and little variability between control and case patients (Ng et al., 2009; Huang et al., 2010). In another case, it has been reported, in serum samples from lung cancer patients, miR-16 being inconsistent, choosing to directly normalize the expression levels of target microRNAs to total RNA (Chen et al., 2008). Several authors have concluded that a universal endogenous control is unlikely to be discovered and a suitable reference should be assessed every time considering the different biological conditions of the samples. However, cost and sample requirements needed for the choice of several reference RNAs are not always possible, especially in a clinical or diagnostic setting.

CONCLUSIONS

As described in the previous paragraphs, microRNAs have some advantages as high specificity, sensitivity, and classification power, which can be exploited for cancer personalized medicine. Furthermore, microRNAs are remarkably stable small molecules shown to be well preserved in FFPE as well as in fresh snap-frozen specimens and in biofluids.

microRNAs affect cancer biology being involved in all the hallmarks of cancer both as tumor suppressor and as onco-miR. They can be extracted from different biological sources and used as biomarkers in order to classify cancerous vs. non-cancerous tissues, distinguish different cancer types and also efficient cancer therapies. Thanks to the sensitive signatures, patterns of microRNAs may be able to follow the progression of cancer, being an important tool in clinical practice. In particular, detection of c-microRNAs, obtained by a non-invasive procedure, seems to be a new promising field with the potential of revolutionizing cancer diagnostics, increasing compliance of patients, ease of use and accessibility to these biomarkers. As a result of a growing use of high-throughput techniques, another emerging field in microRNA diagnostics is represented by SNPs analysis. miRSNP can affect microRNA expression and function, being present on microRNAs sequence, on their target genes or also in genes involved in their biogenesis. They can affect cancer susceptibility, prognosis and response to treatment.

In this review, we reported the main concepts on microRNA cancer personalized medicine. However, some issues have to be

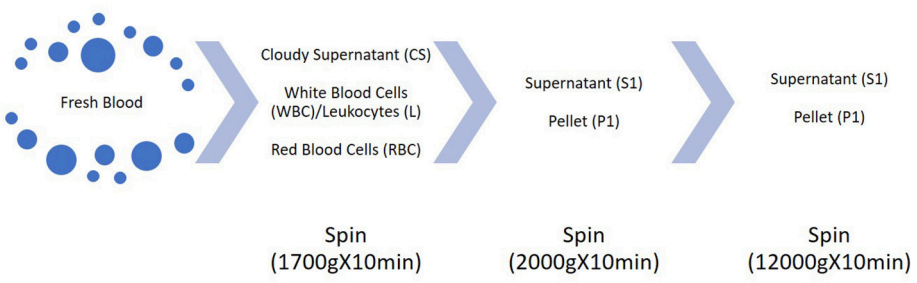

FIGURE 3 | Scheme of the multiple centrifugation steps performed in Duttagupta et al. (2011).

considered while going through the huge amount of studies on microRNA function and classification capacity.

Despite the advances obtained in the field, many open questions and challenges still remain to be addressed (**Table 3**). One limitation in microRNAs detection is represented by the closely similar sequences among microRNAs family members, their ancestral RNAs (pre-microRNAs and pri-microRNAs) and isomiRs (Thomas et al., 2010; Chugh and Dittmer, 2012; Zhou et al., 2012). Moreover, there are many methods which can be used to measure microRNAs including qRT-PCR, microarrays, next generation sequencing (NGS), and more recently, digital droplet PCR (ddPCR). These conventional methods have of course their own drawbacks, mainly time-consuming, expensive material and target modification steps for detection. Concerning c-microRNAs, the main limitations are represented by the small amount of RNA and the corresponding microRNAs extracted from biofluids. Indeed, RNA concentration is often under the detection limits of common spectrophotometric devices and this is the reason why for qRT-PCR, it is recommended to use a fixed volume rather than a fixed RNA amount. Furthermore, the right selection of a suitable normalization method to remove technical variations and increase the accuracy of microRNAs quantification is of enormous relevance. The issue of reference genes is especially critical in the quantification of c-microRNAs, due to the extremely low levels in biofluids of common reference genes (U6, miR-16, 5S rRNA, small nucleolar RNAs).

On the other hand, from a biological point of view, microRNAs biogenesis and function must be further explored in order to better understand mechanisms at the basis of their involvement in cancer. The commonly accepted mechanism of microRNA action and targeting involves the interaction between microRNA 5′-end ("seed region") and mainly the mRNA 3′-UTR. However, target sites were found also in the coding sequence (CDS) and in 5′ UTR (Lytle et al., 2007; Kloosterman et al., 2016). It has been suggested that this preference could be due to the presence of ribosomes in CDS and translation initiation complexes in 5′ UTR which compete with the RISC complex (Bartel, 2004). Regarding the way of action, microRNAs contribute to gene expression regulation by fine-tuning rather than knocking-down their target mRNA. Thus, microRNAs over-expression performed for luciferase assay or western blot, the "gold standard" techniques to analyze their effect on targets, do not represent the real physiological situation. Although microRNAs exert slight effects on mRNAs, they adopt other strategies to potentiate their regulation. Indeed, one microRNA can simultaneously regulate multiple targets and different microRNAs can have a role as post-transcriptional regulators on the same target. In addition, microRNAs are often involved in feedback loops, thereby potentiating their suppression potential. A complex interplay exists also between transcriptional and post-transcriptional regulators (transcription factors and microRNAs) to orchestrate gene expression and signaling. Moreover, some microRNAs are able to regulate gene expression of their own biogenesis and processing factors, as Dicer (Ristori et al., 2015). Lastly, it is important to take into account the tissue specificity of microRNAs action, thus studies on single tissue-type are to be considered carefully. All these interactions and regulation levels lead to highly complex networks of microRNA/target pathways. For this reason, systems biology approach tries to obtain a more comprehensive understanding of miRNA regulatory structure, combining biological data acquisition and integration, network construction, mathematical modeling and experimental validation.

One important point to mention is that, recently, miRNA–target interaction knowledge has been enriched by the discovery of long non-coding RNAs (lncRNAs) (Yoon et al., 2014) and circular RNAs (circRNAs) (Hansen et al., 2013; Memczak et al., 2013) and circRNAs which could act as miRNA sponges, reducing their regulatory effect on mRNAs. One hypothesis is that all RNA transcripts containing binding sites for microRNAs can compete specifically for shared microRNAs, acting as competing endogenous RNAs (ceRNAs) (reviewed in Thomson and Dinger, 2016). This concept is extremely important not only for having a complete view of the mechanisms of action of the microRNAs, but also for their biomarker employment.

In the future new more accurate and PCR-free single base sensitive platform are needed. Therefore, a device in which sample-preparation steps (e.g., enzymatic steps for PCR-based amplifications) are removed, would represent an improvement in microRNAs detection and quantification. Some tentative approaches are under study like the integration of a dynamic chemistry for "Single Nucleobase Labeling" with a bead-based platform (Luminex®) (Venkateswaran et al., 2016), or the use of a power-free microfluidic chip involving a technology based on laminar flow–assisted dendritic amplification (LFDA) (Hasegawa et al., 2017). In the former case the same technology was used to

TABLE 3 | Challenges on microRNA studies from basic microRNA analysis to microRNA functional studies.

microRNA analysis	**Source of preparation:** lack of standardized protocols
	Discrimination between pri-, pre- and mature forms: difficult to distinguish the different forms of microRNA maturation
	Detection techniques: lack of strong sensitivity and sensibility at single-base level
	Short sequence for primer design
	Quantity in biofluids: the low quantity of microRNAs in biofluids demands high-sensitivity techniques
	Normalization methods: the particular nature of microRNAs and their involvement in post-transcriptional regulation makes difficult to find an universal normalizer
microRNA functional studies	**High biological complexity:** their ability to target multiple mRNAs and the presence of multiple different target sites on a single mRNA creates a complex network of regulation difficult to untangle
	Non-physiological conditions: microRNAs generally act as fine-tuners of gene expression, thus, forced over-expression or inhibition in cellular or non-cellular systems are not representative of the reality
	Few mechanicistic studies on miRSNP: miRSNP have been studied mainly for their association to cancer risk but only few of these works try to unravel the mechanism underneath the shown effects

detect microRNAs from serum samples (Rissin et al., 2017). On top of that, new methods to avoid normalization of signals are requested, like the use of ratio between the Ct of two different miRNAs (Sharova et al., 2016). As the detection techiques are fundamental, even the source of microRNAs affect the biological outcome. In this sense, exosomes are representing the future of biomarkers from liquid biopsies. More effort in studying this vehicles will help elucidate the mechanism for which microRNAs are realeased in biofluids, thus affecting our way to use them as biomarkers. In addition, the main step forward toward a safe and stable use in clinics of these tools will be the standardization of protocols regarding pre- and post analytical factors. Therefore, a standardized method for isolation of tissues of biofluids as well as preservation of the sample will drastically decrease variability of results, enhancing similarity and robustness of studies in literature. This point is still missing and it is of critical relevance.

In conclusion, microRNAs are fundamental regulator of cell life, linking all its biological functions. Although their analysis has some challenges, the above-mentioned advantages reveal microRNAs as important tools for biomarkers investigation. If this field will be further pursued, clinicians could be guided by simple tests detecting pattern of microRNAs expression or even single nucleotide variations, making them strongly valid for cancer personalized medicine.

AUTHOR CONTRIBUTIONS

SD designed the work thinking how it should have been organized, drafted and wrote the manuscript, draw the figures and revised the manuscript critically. MG contributed to the conception and design of the work, supporting the revision for important intellectual content. VDV revised the work critically. MD gave overall intellectual critical support and approved the version to be published. All authors read and approved the final manuscript.

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REVIEW
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microRNAs and Acute Myeloid Leukemia Chemoresistance: A Mechanistic Overview

Martino Marco Gabra[1] *and Leonardo Salmena*[1,2]*

[1] Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada, [2] Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada

Up until the early 2000s, a functional role for microRNAs (miRNAs) was yet to be elucidated. With the advent of increasingly high-throughput and precise RNA-sequencing techniques within the last two decades, it has become well established that miRNAs can regulate almost all cellular processes through their ability to post-transcriptionally regulate a majority of protein-coding genes and countless other non-coding genes. In cancer, miRNAs have been demonstrated to play critical roles by modifying or controlling all major hallmarks including cell division, self-renewal, invasion, and DNA damage among others. Before the introduction of anthracyclines and cytarabine in the 1960s, acute myeloid leukemia (AML) was considered a fatal disease. In decades since, prognosis has improved substantially; however, long-term survival with AML remains poor. Resistance to chemotherapy, whether it is present at diagnosis or induced during treatment is a major therapeutic challenge in the treatment of this disease. Certain mechanisms such as DNA damage response and drug targeting, cell cycling, cell death, and drug trafficking pathways have been shown to be further dysregulated in treatment resistant cancers. miRNAs playing key roles in the emergence of these drug resistance phenotypes have recently emerged and replacement or inhibition of these miRNAs may be a viable treatment option. Herein, we describe the roles miRNAs can play in drug resistant AML and we describe miRNA-transcript interactions found within other cancer states which may be present within drug resistant AML. We describe the mechanisms of action of these miRNAs and how they can contribute to a poor overall survival and outcome as well. With the precision of miRNA mimic- or antagomir-based therapies, miRNAs provide an avenue for exquisite targeting in the therapy of drug resistant cancers.

Keywords: microRNA, acute myeloid leukemia, drug resistance, RNA therapy, daunorubicin, cytarabine, chemotherapy

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Technische Universität München, Germany

***Correspondence:**
Leonardo Salmena
leonardo.salmena@utoronto.ca

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INTRODUCTION

Despite rapid progress in our understanding of the cellular and molecular etiology of cancer and the development of countless new anticancer agents and therapeutic strategies, little has changed in the treatment of many cancers over the last few decades. For instance, the standard of care for acute myeloid leukemia (AML) which consists of combined cytarabine and anthracycline therapy has been fundamentally unchanged for the past 30 years (1). The long-standing presence of this strategy is owed to its effectiveness with a mean response rate up to 70% and a lack of superior strategies for most AML subtypes (2, 3). New targeted therapy strategies including monoclonal

antibodies and small molecule inhibitors are constantly being developed; however to date, none of these targeted therapies have proven more effective than the standard of care with the exception of the use of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APL) which has become nearly curable in the majority of cases (4).

Notwithstanding, drug resistance is a major therapeutic challenge in the treatment of AML. Failure of initial therapy can be observed in up to 40% of AML patients, and even when initial therapy is effective, up to 70% of patients eventually succumb to their disease due to aggressive relapse within 5 years (5–7).

The cause of poor long-term survival is primarily drug resistance, which is either intrinsic in patients that fail initial therapy or acquired after chemotherapy through selection or acquisition of mutations (8). Indeed, relapsed AML is often composed of cells that have distinct molecular and cytogenetic characteristics leading to deficiencies or perturbations in various pathways associated with therapeutic resistance including DNA damage response and drug targeting, cell cycling, cell death, and drug trafficking pathways due to increased or altered drug targets are commonly observed (**Figure 1**) (8–10). Consequently, outcomes of relapsed disease are abysmal, which highlights a desperate need for novel therapeutic approaches with potential to overcome or prevent therapeutic resistance.

Non-Coding RNAs (ncRNAs) in AML Therapy Resistance

Among several emerging functions, ncRNAs can act as modulators of gene expression through roles in epigenetics, transcription, translation, as well as homology-dependent post-translational regulation of mRNA transcripts (11). The most widely recognized class of ncRNAs are the microRNAs (miRNAs), which are small 18–24 bp dsRNAs that use cellular RNA-interference machinery to suppress protein expression levels by both degrading or blocking translation of mRNA transcripts (12, 13). It has been convincingly demonstrated in numerous cancers that miRNAs can (1) promote or suppress the development of cancer, (2) be of value in prediction of treatment responses and disease prognosis, and (3) be perturbed as a response to chemotherapy (14, 15).

This review is focused on the small ncRNAs, the miRNAs, in drug resistance; however, long non-coding RNAs (lncRNAs) which are typically >200 bp in length and comprise a large proportion of cellular transcribed RNA have numerous emerging functions in AML pathogenesis (16). lncRNA dysregulation in AML have been reported to have consequences for various cellular processes such as proliferation, survival, and migration (17–19) and have been associated with poor clinical outcome (20–23). Furthermore, lncRNAs signatures associated with well-defined cancer types (24). For instance, *Homeobox (HOX) transcript antisense RNA* (*HOTAIR*) and *HOX antisense intergenic RNA myeloid 1* (*HOTAIRM1*) are substantially upregulated in AML. It was shown in both cell lines and patient samples that the upregulation of *HOTAIR* is specifically associated with indirect upregulation of c-kit through sponging of *miR-193* (20). Recently, doubt has been raised over the prognostic value of *HOTAIR* by Sayad et al.; however, in case–control samples, there was a trend toward clinical significance of *HOTAIR* (25). *HOTAIRM1*, on the other hand, is thought to behave as an endogenous miRNA-sponge for *miR20a*, *miR-20a/106b*, and *miR-125b* and prevents targeting of *ULK1*, *E2F1*, and *DRAM2* as demonstrated in luciferase reporter assays

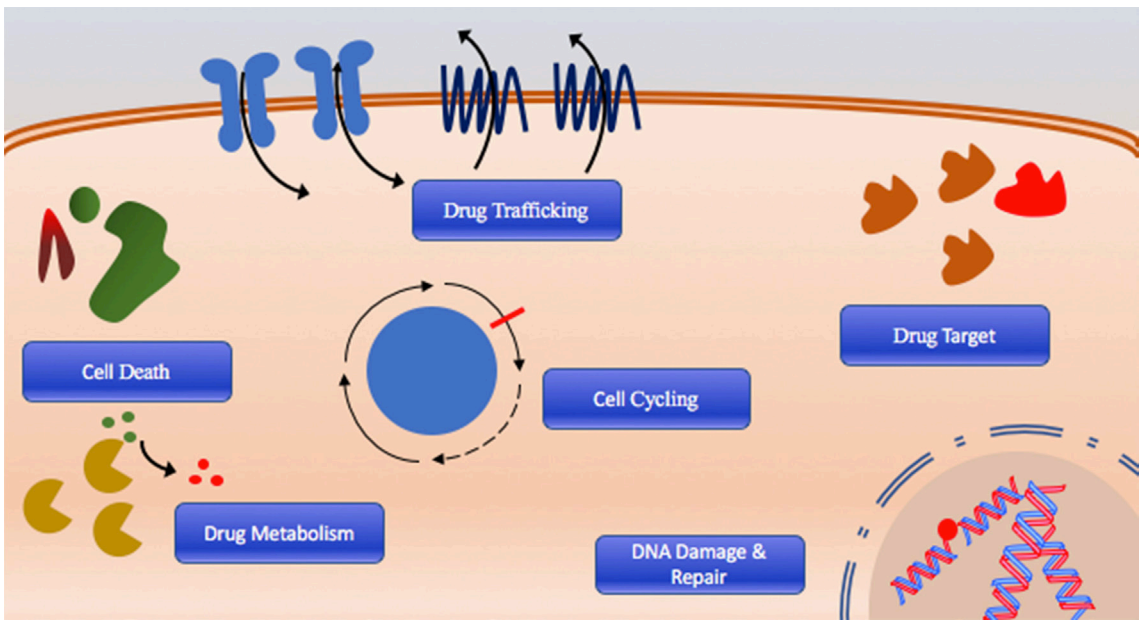

FIGURE 1 | The six hallmarks of drug resistance: DNA damage and repair dysregulation, cell cycle dysregulation, cell death evasion, altered drug metabolism, altered drug target, and dysregulated drug trafficking.

(26, 27). In drug resistant AML, however, little is known about the dysregulation of lncRNAs and their respective mechanisms of function.

miRNA Biology

miRNA derive from the transcription of miRNA loci on genomic DNA by RNA polymerases which create a ~80 nt long transcript primary (pri)-miRNA that are then spliced, capped, polyadenylated, and packaged similar to long-stranded transcripts (28). Further splicing and processing by DROSHA and PASHA transform the pri-miRNA into pre-miRNA. When pre-miRNA exits the nucleus through the function of exportin-5, it is folded into a self-bound hairpin secondary structure known as a "stem-loop" (28, 29). At this stage, the 70–100 nt which make up this stem-loop pre-miRNA is cleaved by a cytoplasmic RNase III such as Dicer into a dsRNA dimer which rapidly breaks down into two strands (29). Depending on the stability of the single strand of miRNA either strand can be active (30–32). A functional third miRNA formed from this complex is thought to originate from the loop region, known as loop-miRNA (33, 34). Next, single-stranded mature miRNAs 19–25 nt in length, bind to the argonaute (Ago) proteins which are one member of a complex of proteins collectively known as the RNA-induced silencing complex (RISC) (35, 36).

Guided by miRNAs, Ago and the RISC move to miRNA recognition elements on mRNA which are commonly, but not limited to non-coding 3′-untranslated regions (3′-UTR) (37, 38). Unlike siRNA, miRNA do not require perfect complementary binding; and only binding to the seed-region appears to be a requirement in most cases (39, 40). This comparatively less stringent binding compared to siRNA allows miRNA to regulate the expression levels of multiple RNA transcripts through target promiscuity (39). Once bound to a target, the endonuclease activity of the RISC is activated *via* the slicer activity of Ago1 (28, 41). Following cleavage, the entire strand is rapidly degraded by endonucleases. Multiple interactions between miRNA and mRNA transcripts are the basis of complex cellular regulatory networks whereby miRNAs control the majority of all protein-coding genes and countless other non-coding genes. In cancer, miRNAs have been demonstrated to play critical roles by modifying or controlling all major hallmarks of cancer including cell division, self-renewal, apoptosis, and DNA damage response among others (42–47).

To date, no comprehensive study examines the role of miRNAs in drug resistant AML. Herein, we describe the miRNAs that have been examined in clinical samples and we highlight miRNA that have been examined mechanistically. Furthermore, we discuss potential miRNA-binding partners of important AML drug resistance machinery found within other cancers to guide future research.

AML CHEMOTHERAPY, DNA DAMAGE, AND miRNA DYSREGULATION

The most common treatment for AML includes an anthracycline like daunorubicin and a nucleoside analog like cytarabine in the "7 + 3" regimen where daunorubicin is administered IV for the first 3 days concomitantly to the IV infusion of cytarabine for 7 days (48, 49). The 7 + 3 regimen is termed *induction therapy* (because of its intent is to induce remission) and has been in place since the 1960s (50). The aim of induction therapy is achieving complete remission (CR), defined clinically as myeloid blast counts in the bone marrow below 5% or minimum residual disease status (49). Efforts to enhance this regimen by escalating dose or adding a third drug has only resulted in increased toxicity with minimal improvement in patient survival. Upon achieving CR, treatment can be consolidated using high doses of cytarabine. Unfortunately, despite undergoing such aggressive chemotherapy regimen with all the associated toxicities and side effect, many patients still relapse within 5 years (48, 49). This is in part due to lack of targeting of leukemic-initiating cells, selection of rare pre-existing resistant AML clones, or the mutagenic effects of the treatments, all of which increase the probability of generating more aggressive AML.

Fundamentally, drug resistance occurs in cells which can evade or withstand treatment. While tumor heterogeneity may explain selection of a pre-existing clone with a favorable mutation, acquired drug resistance is generally defined as the ability of a cell to resist response to the drug to which it was initially responsive. Acquired resistance may be achieved through multiple dosing of the same drug or through as little as a single dose may be explained by the mechanism of drug action.

For instance, anthracyclines used to treat AML such as daunorubicin, doxorubicin, and idarubicin, intercalate DNA, and stall proper DNA replication events (51). Anthracyclines can also target topoisomerase II which normally binds to the scaffold/matrix-associated protein region (S/MAR) to resolve DNA supercoils (52, 53). By binding to topoisomerase II in its open DNA-bound conformation, a stall occurs which can lead to a double-strand break. These double-strand breaks may be fixed aberrantly through non-homologous end joining which can lead to gene mutation. One common mutation in AML, t4:11, occurs at an S/MAR (54–56). This mutation has also been shown to occur in significant proportions in secondary AML patients as well (56). Loss or translocation of the S/MAR may further modulate various miRNAs. As demonstrated by Chavali et al., protein binding to the S/MAR induces histone acetylation that leads to the increased expression of the *miR-17-92* cluster and the miRNAs *miR-221*, *miR-93*, *miR-17*, and *let-7b* (57). As DNA damage is most likely to occur in these regions due to daunorubicin, it is likely that dysregulation of miRNA expression can be due to daunorubicin-induced damage directly.

Cytarabine, on the other hand, is a cytosine analog that terminates translation and replication events. It primarily inhibits cells in S phase (DNA replication) but can also inhibit the progression from G_1 phase into S phase (58, 59). It is known that cytarabine is first metabolized into the triphosphate bound product by deoxycytidine kinase (DCK) and other nucleoside analog enzymes whereby it can then incorporate into the DNA. It is shown that its incorporation can often lead to extensive DNA damage including chromatid breaks (60). Stalled replication forks can also lead to bypass mechanisms such as translesion synthesis (61). This method of DNA replication is more error prone and can lead to mutation events as well. Each of these mechanisms

can be demonstrated to have direct or indirect consequences for miRNA function.

As described with both drugs, genotoxic effects can lead to breaks that are then repaired using homologous or non-homologous repair mechanisms leading to miRNA alterations and the upregulation of drug resistance mechanisms. Conversely, miRNA which regulate these associated pathways may also contribute to drug resistance when perturbed by increasing tolerance to DNA damage. For instance, ataxia telangiectasia mutated (ATM) is an important DNA damage sensing and DNA damage response protein that has been demonstrated to contribute to chemoresistance (62). In experiments conducted in leukemic HL60, NB4, and K562 cell lines, it was found that the overexpression of *miR-181a* leads to increased cell proliferation and increased cell cycling through ATM targeting and downregulation (63). Similarly, *miR-128* was reported to affect the propensity for DNA damage in AML cells. In a study conducted in HL60 cells, it was observed that the transfection of *miR-128* led to increased apoptosis, drug sensitivity, and the amount of DNA damage tolerated; however, the mechanism is yet to be elucidated (64). *miR-128* is thought to be upregulated in various cancers, but its levels are reduced in AML cells carrying *NPM1* mutations (**Figure 2**; **Table 1**) (65, 66).

Recently, Lai et al. identified a mechanism by which *miR-128* is likely targeting *Rad51* directly and leading to the increased DNA damage response in OCI-AML3 and MV4-11 AML cell lines. In these experiments, *miR-128* led to the sensitization of these cell lines to sapacitabine, a novel oral nucleoside analog prodrug (46). In other cancers, *Rad51* has been shown to be a direct target of other miRNAs such as *miR-506*, *miR-103*, and *miR-107*. Clinical significance in chemoresistant high-grade serous ovarian cancers was established for *miR-506* while a miRNA mimic library screen revealed *miR-103* and *miR-107* as strong drug resistance contributors in the U2OS cell line, a model for osteosarcoma (**Figure 2**; **Table 1**) (45, 47). To date, proteins that are thought to be integral to the activity of anthracyclines and nucleoside analogs such as topoisomerase II and the DNA polymerases are not known to interact with miRNAs. However, topoisomerase II has been demonstrated to be downregulated in drug resistant subtypes of AML (67, 68). miRNA targeting may prove to be a mechanism of topoisomerase II downregulation, but more research is required to establish important links of miRNA-induced dysregulation of DNA repair machinery in drug resistant AML.

miRNA AND CELL CYCLING IN AML RESISTANCE

The cell cycle represents a series of events that require the input of various checkpoint proteins known as cyclins and cyclin-dependent kinases (CDK) to proceed into division (69). These proteins, in turn, receive input from DNA damage sensing proteins such as ATM/ATR and CHK1/2 (70, 71). The majority of rapidly dividing cancer cells can be found in one of two major phases: the interphase; which consists of G1, S phase (DNA replication) followed by G2; and the M phase, where cells undergo mitosis. Cell cycle manipulation can be a drug resistance mechanism as cell cycle arrest at different phases or quiescence can lead to chemotherapy

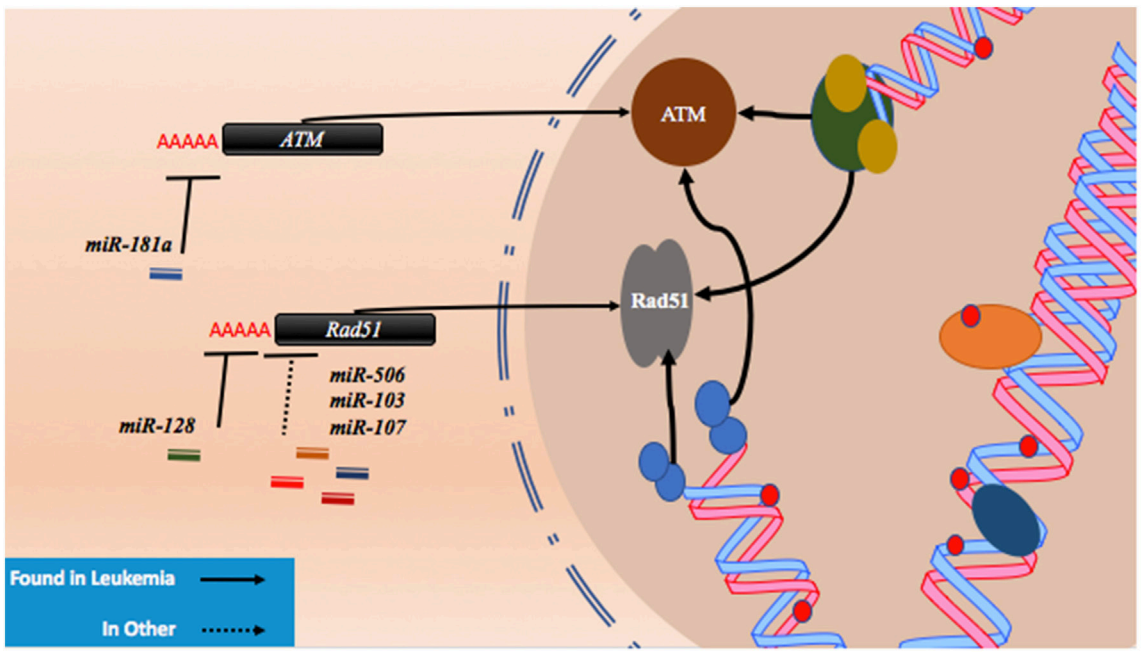

FIGURE 2 | microRNAs (miRNAs) regulate DNA damage response by regulating proteins that behave as DNA damage response elements. In the process of generating DNA damage through genotoxic drugs such as the anthracyclines and the cytosine analogs, the upregulation of effector and response proteins such as ataxia telangiectasia mutated (ATM) and Rad51 is likely to occur. The inhibition of ATM through miR-181a targeting allows tolerance for DNA damage. Reduction of Rad51 through miR-128, miR-506, miR-103, and miR-107 reduces DNA damage response and also contributes to DNA damage tolerance.

TABLE 1 | miRNAs demonstrated to directly bind to DNA damage regulatory proteins.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
ATM	*miR-181a*	Overexpressed	HL60, NB4, K562/AML, CML	ATM downregulation leads to uninhibited growth	Liu et al. (63)
Rad51	*miR-128*	Overexpressed	OCI-AML3, MV4-11/AML	Rad51 downregulation leads to increased DNA damage response	Lai et al. (46)
Rad51	*miR-506*	Overexpressed	Patient samples/high grade serous ovarian cancer	Rad51 downregulation leads to increased DNA damage response	Liu et al. (47)
Rad51	*miR-103*	Overexpressed	U2OS/osteosarcoma	Rad51 downregulation leads to increased DNA damage response	Huang et al. (45)
Rad51	*miR-107*	Overexpressed	U2OS/osteosarcoma	Rad51 downregulation leads to increased DNA damage response	Huang et al. (45)

miRNAs, microRNAs; ATM, ataxia telangiectasia mutated; AML, acute myeloid leukemia.

evasion; however, increased proliferation can also contribute to resistance (72–75).

The process of cell division begins in G1 by the duplication of various proteins, chromatin remodeling, and the verification that the DNA is free of DNA damage. In a healthy cell, if substantial levels of DNA damage are found, ATM/ATR become activated leading to eventual CDK2 inhibition and arrest at the G1/S checkpoint through p21 signaling, where the mechanisms of action of many miRNAs have been elucidated (76). *CDK2* has been found to be inhibited by *miR-638*, where it was demonstrated in HL-60, NB4, and THP-1 that an upregulation of *miR-638* leads to a reduction in cell cycling and a differentiation block in APL (77). The differentiation block was found to occur at the G1/S checkpoint and differentiation inducers like ATRA were found to be more effective in cells with *miR-638* downregulation (77).

CDK2 has been demonstrated to be a target of various miRNAs in cancer including *miR-885-5p*, *miR-372*, and *miR-188* (**Figure 3**; **Table 2**). In contrast to *miR-638* in AML, *miR-885-5p* was demonstrated to play a tumor suppressive role in neuroblastoma by inhibiting *CDK2* and promoting senescence and apoptosis (78). *miR-372* demonstrated targeting of both *CDK2* and cyclin A1, which is highly expressed during S phase. Like *miR-885-5p*, *miR-372* was demonstrated to play a tumor suppressive role as demonstrated in HeLa cells and tissue samples of cervical cancer (79). *miR-188* was demonstrated to directly bind several genes which play a role in cycling such as cyclin D1, cyclin D3, cyclin A2, cyclin E1, CDK2, and CDK4 with varying degrees and it demonstrated modest knockdown of CDK2 relative to the other genes (80). In this study, it was found that the arrest occurs at the G1/S transition and that *miR-188* plays a tumor suppressive role (80).

Other miRNAs such as the *miR-16* family members famously known for downregulation of *BCL2* (**Figure 4**) are also shown to simultaneously directly target several cycling genes such as *cyclin D1*, *cyclin D3*, *cyclin E1*, and *CDK6* (**Figure 3**; **Table 2**). As demonstrated in the A549 cell line by Liu et al., this targeting and likely the targeting of downstream effectors leads to the arrest in G1 and at G1/S, a phenomenon observed by others (81, 85, 86). The targeting of *Cyclin E* has since been demonstrated as playing an important role in certain cancers such as cervical cancer and breast cancer (86–89). The *miR-15* and *miR-16* family may be response elements of E2F1 and as such, may be contributing to a feedback mechanism (90).

The transcription factor *E2F* family may also be a target of miRNAs. *E2F7*, a transcriptional response element gene implicated in cell cycling, is downregulated by *miR-26a* in AML (82). This inhibition in turn reduces *c-myc* transcriptional activation and sequential *miR-17-92* reduced transcription, which has previously been implicated in promoting a differentiation block (82, 91, 92). When active, *miR-17-92* members may be in part directly targeting p21 and promoting cycling, as demonstrated in MLL transformed leukemic cells by Wong et al. (83). The inhibition of *E2F7* may lead to a reduction of miRNAs involved in proliferation such as *miR-25*, *miR-26a, miR-27b, miR-92a*, and *miR-7* thus behaving as a regulatory mechanism (93).

In other instances, miRNAs can behave as direct inhibitors of their own transcriptional repressor thus behaving as autoregulatory elements. It has been demonstrated by Pulikkan et al. that this is the case for *miR-223* and *E2F1* regulation (84). E2F1, an important response element in G1/S, can repress transcription of *miR-223* which in turn can repress *E2F1* (84, 94, 95). The differentiation block observed in APL may be further exacerbated by miRNAs like *miR-223* (**Figure 3**; **Table 2**). The complexity of interactions within miRNA–mRNA networks demonstrates the need for further analyses elucidating the major pathways of feedback and feedforward signaling.

CELL DEATH AND miRNA

In the majority of blast cells that experience sufficient levels of DNA damage upon chemotherapy, programmed cell death (PCD) will become activated. PCD may take the form of apoptosis or autophagy. Apoptosis is characterized by specific changes in morphology such as cell shrinkage and pyknosis (96). Autophagy, on the other hand, is characterized by cellular degradation and the re-introduction of catabolic products into anabolic processes (97, 98). Autophagy can play both a detrimental and a beneficial role in cancer cells and it can also contribute to the generation of leukemia (98–100). Apoptosis, on the other hand, while it is an essential component of normal cell turnover, only its downregulation will often be a major contributor for aberrant cancer growth and its further suppression can lead to drug resistance.

miRNA and BCL2 Family Members

miRNA-associated dysregulation of apoptosis has been observed in drug resistant AML cells. Given that AML is often characterized by aberrant DNA repair and maintenance, tolerance of these damaged lesions is observed through the downregulation of pro-apoptotic markers and damage sensors, or the upregulation of antiapoptotic factors. Of the apoptosis-related families, the BCL2 protein family is the most well described in miRNA

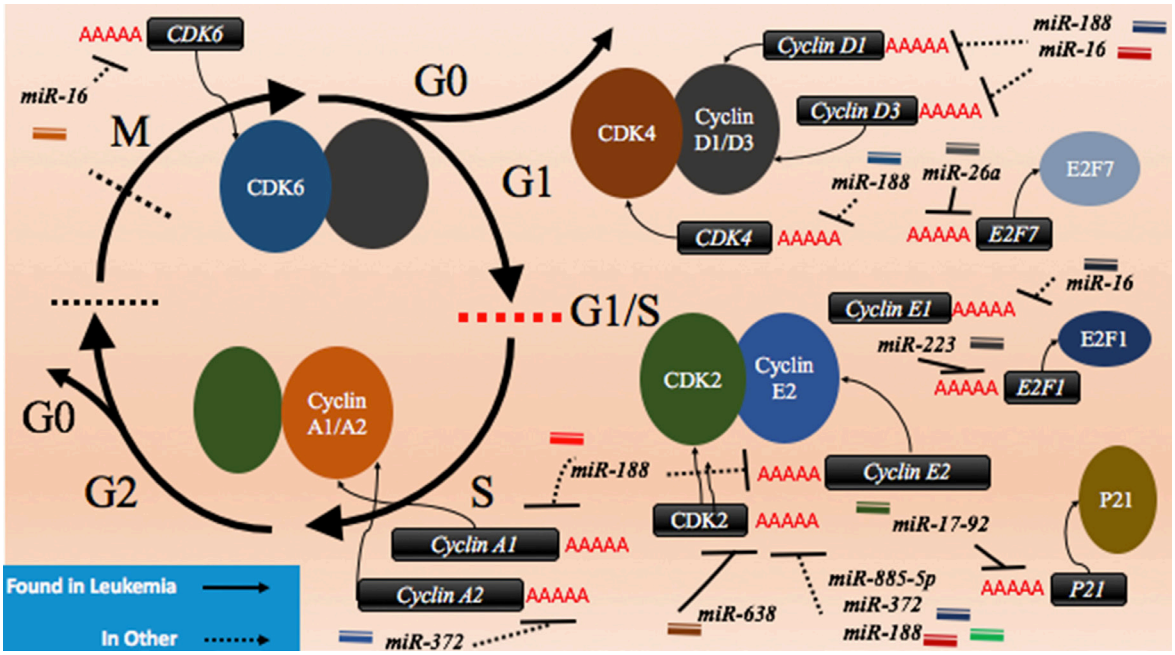

FIGURE 3 | microRNAs (miRNAs) can dysregulate cell cycling mechanisms by dysregulating several phases of the cell cycle, but the majority of known targeting occurs at the G1 and S phases and at the G1/S transition. The downregulation of the cyclins that would normally signal for cell cycling to proceed can be downregulated. Cyclin D1 and cyclin D3 can be dysregulated by miR-188 and miR-16, cyclin E1 can be knocked down by miR-16 while cyclin E2 can be downregulated by miR-17-92 and finally, cyclin A1 and A2 are downregulated by miR-188 and miR-372, respectively. The cyclin-dependent kinases (CDKs) are also adjustable through miRNA targeting and their targeting reduces cycling as well. CDK2 can be downregulated by miR-638, miR-885-5p, miR-372, and miR-188; CDK4 is downregulated by miR-188, and CDK6 is downregulated by miR-16. Effector proteins such as E2F1, E2F7, and p21 can also be downregulated by miRNAs to lead to differentiation blocks. They can be targeted by miR-223, miR-26a, and miR-17-92, respectively.

TABLE 2 | Cell cycling gene dysregulations induced by miRNA binding.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
CDK2	*miR-638*	Overexpressed	HL60, NB4, THP-1/APL	CDK2 downregulation prevents G1/S progression	Lin et al. (77)
CDK2	*miR-885-5p*	Reduction	Patient samples, SH-EP, KELLY, IMR32, SK-N-BE(2)c, and HDN 33 cell lines/ neuroblastoma	Reduced cycling promotes senescence	Afanasyeva et al. (78)
CDK2	*miR-372*	Reduced	HeLa/cervical cancer	Reduced cycling prevents cell growth	Tian et al. (79)
CDK2	*miR-188*	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)
Cyclin A1	*miR-372*	Reduced	HeLa/cervical cancer	Reduced cycling prevents cell growth	Tian et al. (79)
Cyclin D1	*miR-188*	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)
Cyclin D1	*miR-16*	Reduced	A549/lung cancer	G1 and G1/S arrest reduces proliferation	Liu et al. (81)
Cyclin D3	*miR-188*	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)
Cyclin D3	*miR-16*	Reduced	A549/lung cancer	G1 and G1/S arrest reduces proliferation	Liu et al. (81)
Cyclin A2	*miR-188*	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)
Cyclin E2	*miR-188*	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)
Cdk4	*miR-188*	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)
Cdk6	*miR-16*	Reduced	A549/lung cancer	G1 and G1/S arrest reduces proliferation	Liu et al. (81)
Cyclin E1	*miR-16*	Reduced	A549/lung cancer	miR-16 loss may lead to G1 and G1/S arrest reduces proliferation	Liu et al. (81)
E2F7	*miR-26a*	Reduced	Patient samples, HL60, U937/APL	Downregulation of E2F7 reduces progression	Salvatori et al. (82)
P21	*miR-17-92*	Overexpressed	MLL transformed cells/AML	Downregulation of p21 promotes non-differentiation	Wong et al. (83)
E2F1	*miR-223*	Overexpressed	Patient samples, K562, U937/AML, CML	E2F1 downregulation contributes to non-differentiated cell cycle progression	Pulikkan et al. (84)

miRNAs, microRNAs; APL, acute promyelocytic leukemia; CDK, cyclin-dependent kinase; AML, acute myeloid leukemia.

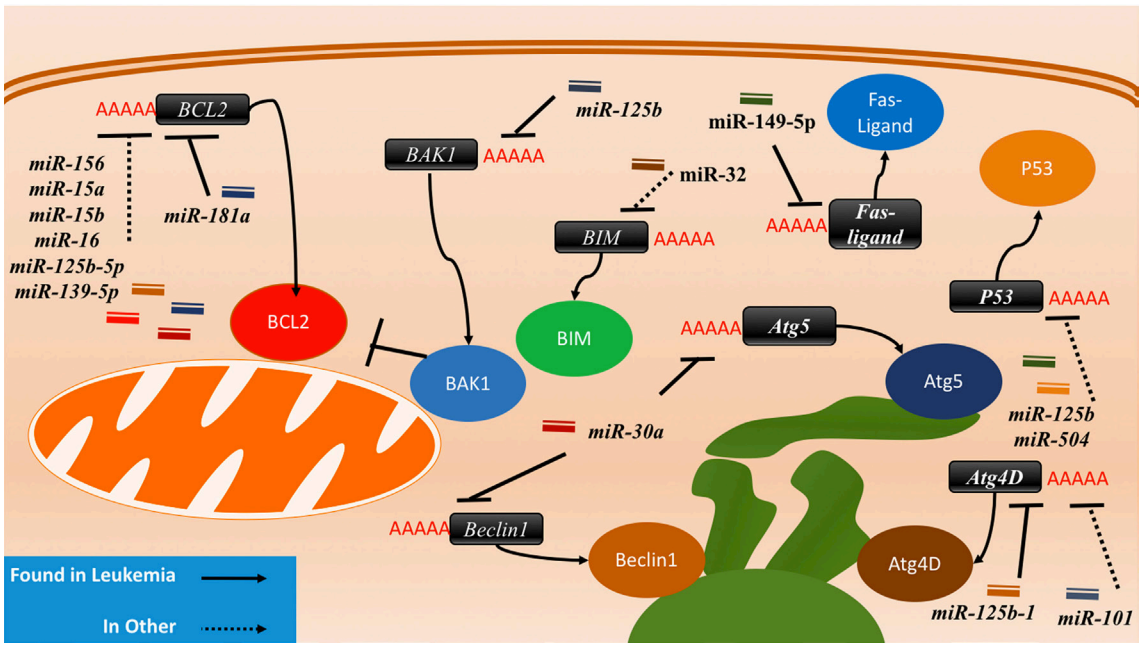

FIGURE 4 | The interactions between microRNAs (miRNAs) and cell death-related proteins in drug resistant cells. Within the apoptosis cell death mechanism, proteins part of the intrinsic or extrinsic pathway can respond to miRNAs to inhibit apoptosis or reduce their regulatory signaling of apoptosis. BCL2, an anti-apoptosis gene, will gain signaling when the associated miRNAs such as miR-156, miR-15a/b, miR-16, miR-125b-5p, and miR-139-5p are lost in the drug resistant cell. The gain of BAK1 miRNA targeting through miR-125b or the gain of BIM targeting through miR-32 will lead to the same effect as well. The Fas-ligand can also be suppressed by miR-149-5p thus ending extrinsic apoptosis signaling. P53 suppression through miR-125b and miR-504 will prevent apoptosis as well. Dysregulating autophagy through increased targeting may increase drug resistance through the binding of miR-125b and miR-101 on Atg4D. miR-30a is known to inversely correlate with Beclin1 and Atg5 in leukemia cell lines, but less is known about the outcome of this interaction.

dysregulation driven in AML. The BCL2 protein itself is commonly considered as a crucial anti-apoptosis gene as it inhibits the mitochondrial pro-apoptotic proteins such as Bak and Bax. While it can be dysregulated or mutated in cancers, it is observed that dysregulation may also occur in the development of drug resistance. Many miRNAs including *miR-15/miR-16*, *miR-125b-5p*, *miR-139-5p*, *miR-145*, and *miR-181a* have been shown to suppress the translation of *BCL2* and decrease the propensity for activation of apoptosis (**Figure 4**; **Table 3**).

Of the *BCL2*-targeting miRNAs, only *miR-181a* has been shown to do so in AML cells. In K562 CML cells, it was demonstrated by Li et al. that the drug resistant form had 40% of the *miR-181a* levels found in the parental cell line. When the parental cells were transfected with a *miR-181a* inhibitor, resistance developed (101). In a separate study conducted by Bai et al. in cytarabine resistant HL60, it was found that the resistance phenotype can be also be attributed to reduced *BCL2* targeting by *miR-181a*, whereas its ectopic expression sensitizes the cells to treatment to cytarabine (102). Other studies of *miR-181a* in AML have also demonstrated that it is often downregulated in drug resistant AML, that it can serve as an independent prognostic marker and potentially modulate the interaction with natural killer cells as well (118–122). In molecular poor risk group AML with *FLT3-ITD* mutations, it was demonstrated that high *miR-181a* also strongly predicted better survival (123).

The *miR-15/16* have been shown to suppress BCL2 in multiple cancers including gastric cancer, breast cancer, and glioma and the loss of this locus has also been observed in CLL (124–127). Xia et al. demonstrated that *miR-15b* and *miR-16* are lost in vincristine resistant SGC7901 cells, a gastric cell line (103). Cittelly et al. later demonstrated that in a common mutation of the *HER2* gene, *HERΔ16*, representative of 30% of HER2 dysregulations in estrogen receptor positive breast cancers, the downregulation of *miR-15a* and *miR-16* is observed (104). In MCF-7 cells ectopically expressing this mutant variant, it was shown that tamoxifen resistance may be in part due to the reduced regulation of *BCL2* by *miR-15a* and *miR-16*, which leads to apoptosis evasion (104). In glioma cells that are resistant to temozolomide, it was demonstrated that the loss of *miR-16* specifically can contribute to resistance in the U251MG/Temozolomide resistant cell line and that the blocking of *miR-16* in the temozolomide sensitive AM38 cell line increased resistance by de-repressing *BCL2* (105).

In a genome-wide gene expression analysis of gallbladder cancer clinical samples, *miR-125b-5p* was found to be statistically downregulated in cisplatin resistant patients ($N = 6$). Analyses demonstrated that this miRNA can directly bind to the 3′UTR of *BCL2*, contribute to cisplatin desensitization, and increase tumor formation in mice (106). A similar analysis of patient samples conducted in colorectal cancer demonstrated that *miR-139-5p* inhibits the epithelial-to-mesenchymal transition and contributes to drug resistance by downregulating *BCL2* (107). Bioinformatic studies also demonstrate binding of other miRNAs to the *BCL2* mRNA as putative mechanisms of miRNA-induced downregulations. For instance, bioinformatic analysis of *miR-451*

TABLE 3 | The interactions of miRNAs with cell death-related proteins.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
BCL2	*miR-181a*	Reduced	K562/CML HL60/APL	Reduced miR-181a leads to increased apoptosis suppression Cytarabine resistance presents with reduced miR-181a expression and apoptosis suppression	Li et al. (101) Bai et al. (102)
BCL2	*miR-15b*	Reduced	SG7901 cells/gastric cancer	Reduced miR-15b expression leads to BCL2 overexpression and apoptosis suppression	Xia et al. (103)
BCL2	*miR-16*	Reduced	SG7901 cells/gastric cancer ERΔ16 MCF7/breast cancer U251MG, AM38	Reduced miR-16 expression leads to BCL2 overexpression and apoptosis suppression	Xia et al. (103) Cittelly et al. (104) Han and Chen (105)
BCL2	*miR-15a*	Reduced	HERΔ16 MCF7/breast cancer	Downregulated miR-15a leads to BCL2 overexpression	Cittelly et al. (104)
BCL2	*miR-125b-5p*	Reduced	Patient samples/gallbladder cancer	Downregulation of miR-125b-5p disinhibits BCL2 and leads to anti-apoptosis	Yang et al. (106)
BCL2	*miR-139-5p*	Reduced	Colorectal cancer	Downregulation of miR-139-5p leads to BCL2 disinhibition and anti-apoptosis	Li et al. (107)
BAK1	*miR-125b*	Overexpressed	HL60, NB4/APL NB4, K562/CML MDA-MB-435, MDA-MB-231/breast cancer HMLE/breast cancer PC-3466C, LNCaP/prostate cancer	Suppression of Bak1 leads to apoptosis avoidance	Zhang et al. (7) Li et al. (108) Zhou et al. (109) Shi et al. (110)
BIM	*miR-32*	Overexpressed	LNCaP/prostate cancer	Downregulation of *BIM* leads to apoptosis evasion	Gocek et al. (111)
p53	*miR-125b*	Overexpressed	SH-SY5Y/neuroblastoma	Direct binding to the *P53* by *miR-125b* leads to further inhibition of apoptosis response	Le et al. (112)
p53	*miR-504*	Overexpressed	HCT116 (colorectal carcinoma), H460 (large cell lung cancer), MCF-7 (ER + breast cancer), U2OS (osteosarcoma), A498 (kidney carcinoma)	Direct binding by *miR-504* reduces the propensity of a cell to enter apoptosis	Hu et al. (113)
Fas-ligand	*miR-149-5p*	Overexpressed	THP-1/AML	Downregulation of the *Fas-ligand* reduces activation of the extrinsic apoptosis pathway	Tian and Yan (114)
Beclin 1	*miR-30a*	Unknown	K562/CML	Inverse correlation found	Yu et al. (115)
ATG5	*miR-30a*	Unknown	K562/CML	Inverse correlation found	Yu et al. (115)
ATG4D	*miR-125b1*	Overexpressed	NB4/APL	Inhibition of autophagy	Zeng et al. (116)
ATG4D	*miR-101*	Overexpressed	MCF7/breast cancer	Inhibition of autophagy contributed to tamoxifen resistance	Frankel et al. (117)

miRNAs, microRNAs; APL, acute promyelocytic leukemial; AML, acute myeloid leukemia.

through miRBase and miRanda identified it as an inhibitor of *BCL2* (128). Similarly, in paclitaxel-resistant breast cancer, it was demonstrated that *miR-451* may also inhibit BCL2.

The BCL2 antagonist/killer 1 (Bak1) protein is upregulated in the progression of apoptosis in normal cells; in drug resistant cancers, however, it is observed that there is *Bak1* suppression through *miR-125b* binding. The binding of *miR-125b* to the *Bak1* transcript was initially examined in the prostate cancer cell lines PC-346C and LNCaP in the context of androgen-independent signaling, but effect on drug resistance was not examined (110). In APL, *miR-125b* was demonstrated to be clinically relevant, in CML mice models, and it was further demonstrated that direct suppression occurs in the cell lines NB4, HL60, and K562 (7, 108). A similar link between *miR-125b* and *Bak1* was established in MDA-MB-435 and MDA-MB-231 where it was demonstrated that *miR-125b* is capable of *Bak1* suppression in Taxol resistant cells (109). The mechanism of *miR-125b* upregulation was further elucidated to be through Wnt signaling and specifically through Snail binding; an upregulation thought to also occur in cancer stem cells (129).

The Bcl-2-like protein 11, also known as, BIM, has been demonstrated to be a direct target of *miR-32* in a previous study in LNCaP prostate cancer cells. This pro-apoptotic protein can be downregulated by *miR-32* and consequently lead to resistance and increased cell proliferation (130). Studies in the AML cell lines HL60 and U937 also demonstrated an inverse correlation between *miR-32* and *BIM* (111).

miRNA and P53 Regulation

The tumor-suppressor protein p53, often referred to as guardian of the genome is dysregulated in 50% of all cancers. In *wild-type* cells, p53 is often suppressed and destabilized by mdm2, mdm4, and mdmx which behave like E3 ligases, marking P53 by ubiquitination for degradation. Phosphorylation of p53 by ATM leads

to its stabilization and release from the mdm protein family. p53 can then behave as a transcription factor by activating apoptosis-related genes (both intrinsic and extrinsic), cell cycle arrest related genes or DNA repair related genes and it can directly bind to the mitochondria to participate in membrane permeabilization (131, 132).

P53 has been identified as a direct target of miRNA binding by *miR-125b* and *miR-504. miR-125b* was shown to directly decrease P53 transcript levels and consequently decrease apoptosis response to irradiation in neuroblastoma cells and in lung fibroblasts (**Figure 4**; **Table 3**) (112). *miR-504* was first computationally predicted and then demonstrated in various cell lines including HCT116 (colorectal carcinoma), H460 (large cell lung cancer), MCF-7 (ER + breast cancer), U2OS (osteosarcoma), and A498 (kidney carcinoma) cells to directly target the 3′UTR of *P53* (113). *P53* is also importantly downregulated through indirect ways by *miR-34a*, which is thought to play a crucial role in P53's pro-apoptotic abilities (133, 134). It has been demonstrated that *miR-34a* can indirectly increase P53 by inhibiting P53 negative regulators such as *SIRT1* in colon cancer as demonstrated by Yamakuchi et al. and likely through binding of *mdm4* as well, as predicted bioinformatically (135–137).

Furthermore, it has been demonstrated that P53 transcriptionally activates *miR-34a* which in turn modulates and fine tunes P53's signal (134). Consequently, the relationship between *miR-34a* and P53 is context dependent as the mutation status of *P53* can influence the response and outcome of *miR-34a* activity (138). In the study conducted by Rücker et al., it was found that P53 alterations were the most common molecular lesions which coincided with complex karyotypes in AML (138). Low *miR-34a* and P53 alterations were shown to have the poorest clinical outcome in terms of drug resistance and survival. The low expression was shown to also correlate with a specific gene expression profile consisting of P53-associated proteins. In complex karyotypes that did not have a *P53* alteration, high *miR-34a* predicted a poor overall survival while loss of *P53* and high *miR-34a* predicted better outcome (138). The interplay between *miR-34a* and *P53* demonstrates that the same miRNA can have opposite effects depending on the mutation status of the associated mRNA and highlights the necessity of describing miRNA activity in relation to the activity of associated mRNA.

Other Apoptosis-Related Proteins

For the apoptotic extrinsic pathway, it was reported by Tian et al. that *miR-149-5p* can directly downregulate the *Fas-ligand* and reduce the levels of the apoptosis effector proteins caspase-8, caspase-2, and caspase-3; however, no effect on drug resistance is demonstrated (114). It is possible that *miR-181a* and *miR-21* can suppress the Fas-ligand in cancers as they are shown to interact with the Fas-ligand in bone marrow-derived mesenchymal cells and cardiomyocytes, respectively (139, 140). The binding of miRNAs to caspases has also not been examined closely in cancers, but in an experiment conducted by Zhang et al. in endothelial cells demonstrated *caspase-3* downregulation due to *let-7g* inhibition. As such, this targeting reduced the progression of apoptosis and lead to higher tolerance of oxidative stress (141).

Autophagy and miRNA

Autophagy is regulated by many autophagy related (ATG) proteins which play various roles in the formation of the autophagosome (100, 142). It has been observed that miRNAs can likely play a role in autophagy and that AML cells can have dysregulated autophagy (97). To date, two miRNAs have been found to associate with autophagy in leukemia: *miR-30a* and *miR-125b1* (115, 116). *miR-30a* is inversely correlated with *Beclin1* and *ATG5* in K562, but direct binding and relevance to drug resistance is yet to be demonstrated (115). *miR-125b1*, on the other hand, can bind *RAM2*, *ATG4D*, and *UVRAG* as demonstrated in NB4 cells (116). The activity of *miR-125b1* in this circumstance contributed to inhibition of autophagy through *ATG4D*. In other cancers, *ATG4D* was found to be a direct target of *miR-101* and its inhibition may contribute to 4-hydroxytamoxifen sensitization in the breast cancer lines MCF7 and T47D (117).

DRUG METABOLISM AND CHEMORESISTANCE

Drug activation and drug clearance can be altered in cells to reduce the effective dose of the drug. These proteins are highly varied, but can largely be characterized into two major classes: the phase I and the phase II class of enzymes. Phase I enzymes typically perform redox reactions or hydrolysis reactions. While they often precede phase II enzyme activity, this is not always required. Phase II enzymes typically increase the polarity of the molecule through the addition of a sub-group such as UDP-glucoronate, sulfate, methane, acetate, or glutathione (143).

Anthracyclines are active drugs that can carry out their genotoxic effects directly. Their metabolism into the semiquinone form, the hydroxyaglycone form, deoxyaglycone form, or the alcohol form will decrease its likelihood of intercalating DNA as it reduces the anthracycline's lipophilicity. It is unclear whether the anthracyclines lose efficacy through metabolism. As demonstrated from cardiotoxicity assays in rat and rabbit, the metabolites may have differing effects depending on the organism in question and the rate of metabolism. In rats, the alcohol form may retain some activity, but the effects of the active drug are more pronounced (143, 144). In rabbits, the alcohol derivative is implicated in the cardiotoxic effects of the anthracyclines (143, 145). It is thought that the enzymes CBR1/3 and AKR1A1/C3 can act on the parent drug to form the alcohol form. The hydroxaglycone and the deoxyaglycone forms can be generated in part by certain cytochrome P450 (CYP) enzymes such as CYP3A4/5, CYP2D6, xanthine dehydrogenase (XDH), and NAD(P)H quinone dehydrogenase 1 (NQO1) (146–150). XDH, NQO1 along with nitric oxide synthase can help in generating the semiquinone form (151–153).

Cytarabine and other nucleoside analogs require phosphorylation through DNA/RNA synthesizing enzymes such as the nucleoside kinases to become candidates for incorporation into nascent DNA. Cytarabine requires activation by several enzymes including deoxycytidine monophosphate kinase, nucleoside

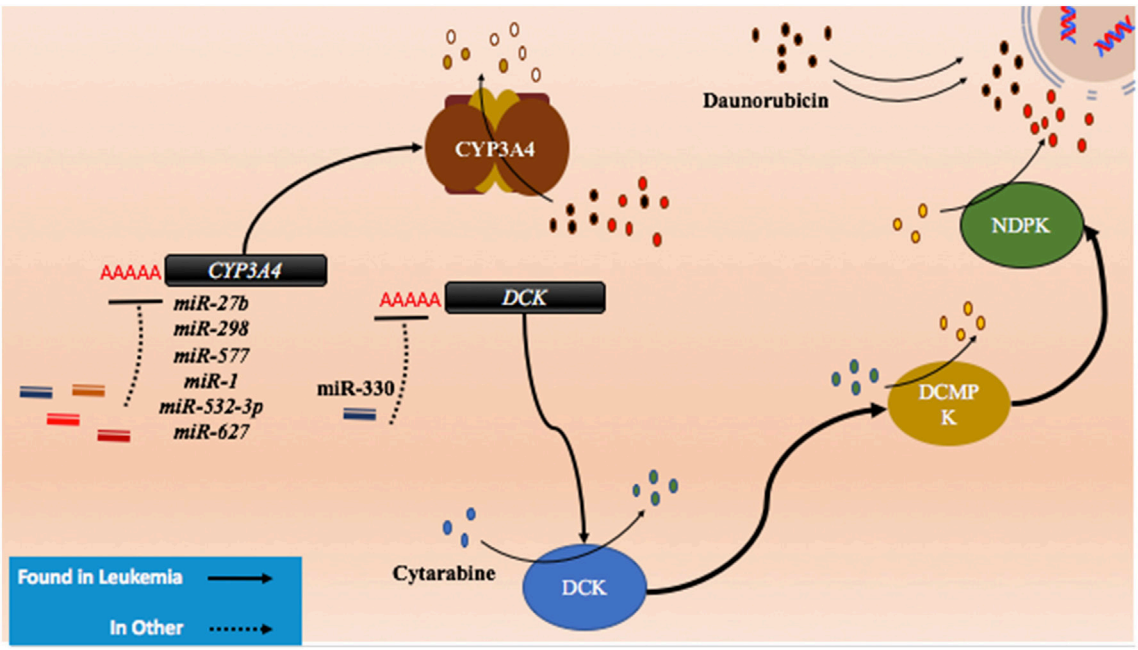

FIGURE 5 | The role of metabolism and microRNA (miRNA) in daunorubicin and cytarabine treatment. While daunorubicin is an active drug, cytosine requires bio-activation. As a cytosine analog, it must undergo three phosphorylation steps to become fully activated and capable of incorporating into the genome. The deactivation of daunorubicin and cytarabine is partially dependent on the cytochrome P450s and they commonly share CYP3A4 in their pathway of degradation. In other cancer, CYP3A4 has been shown to be targeted by miR-27b, miR-298, miR-577, miR-1, miR-532-3p, and miR-627. In the pathway of cytarabine activation, deoxycytidine kinase (DCK) has been shown to be downregulated by miR-330 in other cancers.

TABLE 4 | miRNA targeting proteins involved in drug metabolism.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
CYP3A4	*miR-27b*	Overexpressed	LS-180, PANC 1/colon adenocarcinoma and pancreatic cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Pan et al. (156)
CYP3A4	*miR-298*	Overexpressed	LS-180, PANC 1/colon adenocarcinoma and pancreatic cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	*miR-577*	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	*miR-1*	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	*miR-532-3p*	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	*miR-627*	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
DCK	*miR-330*	Overexpressed	HEK 293T/cancer	Inverse correlation between miRNA-mRNA suggests interaction	Hodzic et al. (158)

miRNAs, microRNAs; DCK, deoxycytidine kinase.

diphosphate kinase, and the rate limiting DCK (**Figure 5**) (154, 155). It is then metabolized by various enzymes including CYP3A4, 5′ nucleotidase, cytidine deaminase, and deoxycytidylate deaminase (154, 155).

Currently, there are few publications that highlight the role of miRNAs in anthracycline and cytosine analog metabolizing enzymes in AML. However, certain miRNAs such as *miR-27b* and *miR-298* have demonstrated direct binding of *CYP3A4* in a pancreatic cell line and *miR-577, miR-1, miR-532-3p*, and *miR-627* were found to target *CYP3A4* in HEK 293T cells (**Figure 5**; **Table 4**) (156, 157). In gemcitabine resistant colon and lung cancer cells, Hodzic et al. established a correlation between *miRNA-330* and *DCK* expression levels (158). Further studies interrogating the role of computationally predicted miRNAs and miRNAs discovered in other cancer subtypes may help establish a role for miRNAs in metabolism in drug resistant AML.

DRUG TRAFFICKING AND miRNA IN CHEMORESISTANCE

The trafficking of the anticancer drugs can dramatically modulate treatment response as a reduction in influx or an increase in efflux will reduce the effective intracellular concentration of drug. Due to the lipophilicity of the anthracyclines, they can freely diffuse into the cell, but they can also bind to the SLC22A16 solute pump to enter cells (153, 159–161). While there are some reports that suggest the role of SLC22A16 in bleomycin resistance, the role of this transporter in anthracycline resistance is yet to be explored (162, 163). As such, while there are predicted miRNA-binding sites on this protein, none are yet confirmed.

Cytarabine and other cytosine analogs, on the other hand, necessitate the function of nucleoside transporters to enter the cell. The nucleoside transporters are composed of six major protein families: human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs), organic anion transporters, organic cation transporters, peptide transporters, and the multidrug resistance protein family (MRP), with the hCNTs and hENTs playing the most major role of cytarabine import (164–166). In childhood leukemia, the hENT protein family has demonstrated to correlate with cytarabine resistance, but miRNA-mediated mechanisms are yet to be confirmed (167, 168).

In contrast, many efflux pumps can confer resistance to diverse and seemingly unrelated drugs and the characterization of several of these transporters has been extensive in AML. These ATP-binding cassette (ABC) proteins can be upregulated in the drug resistant forms of cancers and as such, the downregulation of miRNAs that target efflux pumps can contribute to resistance. Within this class, ABCB1 (P-glycoprotein, MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2 (BCRP) have been the most extensively examined out of 48 proteins within this functionally similar class (**Figure 6**) (169, 170). Indeed, previous treatments of drug resistant AML centered on the targeting of P-glycoprotein. It has been clearly demonstrated that the surface expression of P-glycoprotein is inversely proportional to the concentration of intracellular daunorubicin in blast cells and in tissue culture samples; however, blocking of P-glycoprotein did not yield positive results in clinical settings (171).

P-glycoprotein can be targeted by several miRNAs including *miR-27a, miR-331-5p, miR-145, miR-298, miR-508-5p, miR-9,* and *miR-451* (**Figure 6**; **Table 5**). In leukemia, only *miR-27a* and *miR-331-5p* have been demonstrated to bind to *P-glycoprotein* in the K562 and HL-60 leukemia cell lines (172). In ovarian and cervix cell lines, it was demonstrated that the downregulation of both *miR-27a* and *miR-451* can lead to downregulation of P-glycoprotein; however, in the case of *miR-27a*, this contradictory effect on P-glycoprotein is likely in part due to targeting of *HPK2* upstream (173). This was further phenotypically demonstrated by the reduced uptake of intracellular dyes and by the response to cisplatin and methotrexate (174). In more recent experiments conducted in hepatocellular carcinoma cells, in addition to direct binding to *P-glycoprotein* and *HPK2* binding, it was demonstrated that the inhibitory effect of *miR-27a* on *P-glycoprotein* may also be partially attributed to upstream modulation of the β-catenin pathway through direct binding of

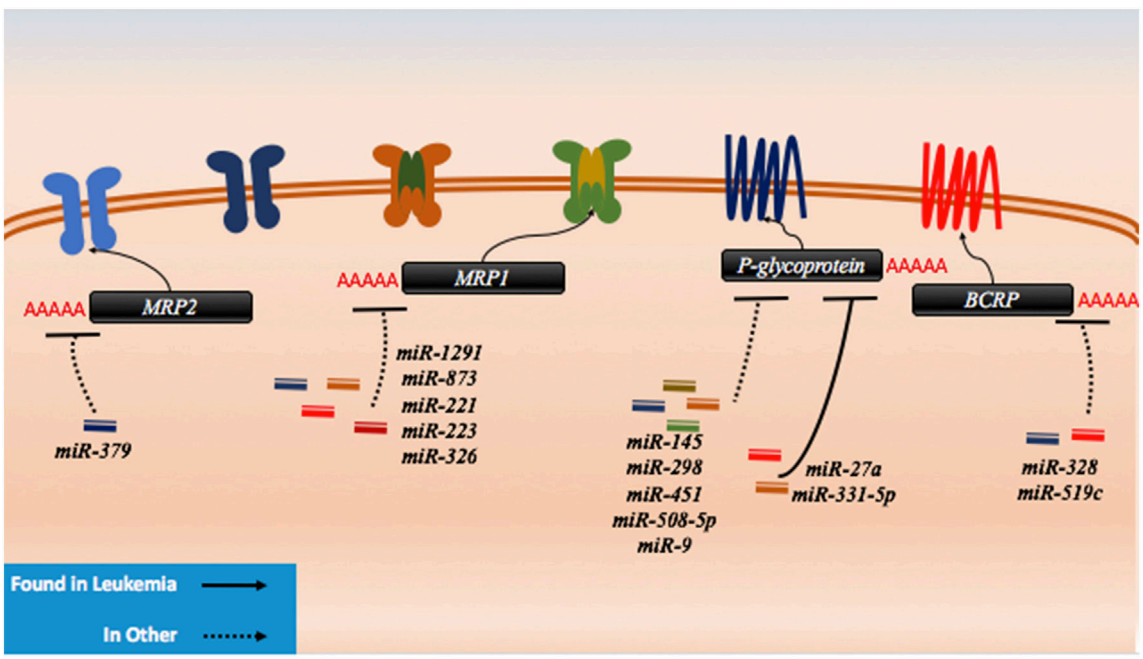

FIGURE 6 | microRNAs (miRNAs) have been shown to dysregulate drug efflux mechanisms in both leukemia and other cancer. There are no known miRNA regulators of the drug influx proteins. In leukemia, P-glycoprotein has been demonstrably targeted by miR-27a and miR-331. In other cancers, P-glycoprotein has been shown to be regulated by miR-145, miR-298, miR-451, miR-508-5p, and miR-9. MRP1 has been targeted by miR-1291, miR-873, miR-221, miR-223, and miR-326, while MRP2 has been shown to be targeted my miR-379. The last protein to exhibit miRNA binding in lab setting is BCRP which has been shown to be a target of miR-328 and miR-519c.

TABLE 5 | Drug trafficking gene disinhibitions caused by loss of miRNAs can lead to drug resistance.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
ABCB1 (P-glycoprotein/MDR1)	*miR-145*	Reduced	Caco2 cells, HEK293/colorectal adenocarcinoma	Reduced efflux leads to multidrug resistance	Ikemura et al. (177)
ABCB1 (P-glycoprotein/MDR1)	*miR-298*	Reduced	MDA-MB-231/breast cancer	Reduced efflux leads to multidrug resistance	Bao et al. (178)
ABCB1 (P-glycoprotein/MDR1)	*miR-27a*	Reduced	K-562, HL60, patient sample/AML A2780/ovarian cancer A2780, KB-3-1/ovarian cancer	Reduced efflux leads to multidrug resistance	Feng et al. (172) Li et al. (173) Zhu et al. (174)
ABCB1 (P-glycoprotein/MDR1)	*miR-331-5p*	Reduced	K-562, HL60, patient sample/AML A2780/ovarian cancer	Reduced efflux leads to multidrug resistance	Feng et al. (172)
ABCB1 (P-glycoprotein/MDR1)	*miR-451*	Reduced	A2780/ovarian cancer MCF-7 cells/breast cancer	Reduced efflux leads to multidrug resistance	Li et al. (173) Kovalchuk et al. (179)
ABCB1 (P-glycoprotein/MDR1)	*miR-508-5p*	Reduced	SGC7901/gastric cancer	Direct binding leads to reduced efflux and to multidrug resistance	Shang et al. (175)
ABCB1 (P-glycoprotein/MDR1)	*miR-9*	Reduced	U87 and T98G/glioblastoma multiforme	Putative or indirect knockdown. Reduced efflux leads to multidrug resistance	Munoz et al. (180)
MRP1	*miR-1291*	Reduced	PANC1/pancreatic cancer	Loss of binding of MRP1 contributes to doxorubicin resistance	Pan et al. (181)
MRP1	*miR-873*	Reduced	OVCAR3 and A2780/ovarian cancer	Loss of binding of MRP1 contributes to multidrug resistance	Wu et al. (182)
MRP1	*miR-221*	Reduced	NCI-H929, RPMI-8226, and U266/multiple myeloma	Loss of binding leads to MRP1-mediated drug resistance	Gullà et al. (183)
MRP1	*miR-222*	Reduced	NCI-H929, RPMI-8226, and U266/multiple myeloma	Loss of binding leads to MRP1-mediated drug resistance	Gullà et al. (183)
MRP1	*miR-326*	Reduced	MCF7/breast cancer	Inverse correlation, and likely binding of miRNA	Liang et al. (184)
MRP2	*miR-379*	Reduced	HepG2/hepatocellular carcinoma	Reduced miR-379 binding leads to MRP2 overexpression and increased efflux	Haenisch et al. (185)
BCRP	*miR-328*	Reduced	MCF7/breast cancer	Inverse correlation of the miRNA-mRNA pair, suppression of BCRP is possible and it is leading to resistance	Pan et al. (186)
BCRP	*miR-519c*	Reduced	S1/colon cancer	Transcript variant of BCRP loses miR-519c binding site to lead to resistance	To et al. (187)

miRNAs, microRNAs; AML, acute myeloid leukemia.

SFRP1 and potentially through *FZD7* as well (172, 175). It is possible and likely that P-glycoprotein is involved in processes that are unrelated to drug trafficking as well such as apoptosis which may explain the contradictory expression in different cancers and the varying predisposition of its mutagenicity in certain cancers; however, its actions remain unclear (176).

Direct binding of *miR-451* to *P-glycoprotein* transcripts was demonstrated in MCF-7 cells, where it was demonstrated that it could contribute to doxorubicin resistance; however, this has not yet been demonstrated to be clinically significant in cancer patients (179). In colon cancer cell-derived cell lines and HEK293 cells, it was demonstrated that *miR-145* can play a role in the repression of P-glycoprotein and increase the efflux of rhodamine 123 (177). *miR-298* was demonstrated to directly bind to the transcript in resistant breast cancer cell lines (178). This suggests that it may play a role in patients, but follow-up studies are needed. *miR-508-5p* was demonstrated to directly bind to *P-glycoprotein* in gastric cancers and its upregulation was found clinically as well (175). It has also been suggested by Munoz et al. that *miR-9* may also target P-glycoprotein and confer resistance to temozolomide in glioblastoma multiforme cells (180). These miRNAs may also prove to be relevant in AML, but no studies have been attempted to date.

While the *MRP1* gene has not demonstrated miRNA binding in AML, it was demonstrated in other cancers that the *MRP1* gene can also be targeted by miRNAs such as *miR-1291, miR-873, miR-221/222,* and *miR-326* (**Figure 6**; **Table 5**). In an analysis conducted by Pan et al., doxorubicin treatment of pancreatic cancer cells demonstrated that *miR-1291* will become upregulated and target *MRP1* directly (181). *MRP1* downregulation contributes to multidrug resistance as well in other cancers such as ovarian cancer (182). It was recently demonstrated through *in vivo* and *in vitro* studies that *miR-873* can be biologically significant in paclitaxel and cisplatin resistance in ovarian cancer cell lines

where it can directly bind to *MRP1* (182). Consequently, *miR-873* is often downregulated in *MRP1*-dependent ovarian cancers. In melphalan-refractory multiple myeloma cells, Gulla et al. demonstrated that *miR-221/222* may be binding and reducing *MRP1* thus contributing to drug resistance (183). Finally, *miR-326* was inversely correlated with *MRP1* in multidrug resistant MCF7 cell lines (184). Less is known about *MRP2* targeting by miRNAs, but in the liver cell line HepG2, *miR-379* was demonstrated to be highly upregulated and to target *MRP2* directly as a response to Rifampicin resistance (185).

BCRP, in contrast, has been shown to be a target of *miR-520h, miR-328*, and *miR-519c* and to potentially play a role in the hematopoietic system (**Figure 6**; **Table 5**). In $CD34^+CD38^-$ hematopoietic stem cells, it was demonstrated that *miR-520h* is enriched compared to $CD34^+$ cells alone and that it can directly target *BCRP* in this fraction (188). An examination of *miR-520h* in leukemic cells and AML may demonstrate a similar trend of upregulation and a contribution of *miR-520h* to drug resistance, but more experiments are required. In mitoxantrone-resistant MCF-7 cells, Pan et al. showed that the expression of *miR-328* is inversely correlated with *BCRP* and that it is directly suppressing *BCRP*, leading to resistance (186). To et al. demonstrated that *miR-519c* may play a role in downregulating BCRP in S1 colon cancer cell lines; however, they demonstrated that binding of *miR-519c* was limited to a longer form of the transcript only found in their parental cell line compared to their mitoxantrone-resistant counterpart (187, 189). This study highlights the importance of splice variants and how they may gain or lose miRNA-binding sites and thereby contribute to resistance.

IMPLICATIONS IN TREATMENT

Drug resistance is only a single aspect of clinical setbacks; however, it is a major contributor to therapy failure. Although treatment has improved substantially in some cancers in the past few decades, many other cancer types continue to demonstrate substantial patient populations that relapse after an initially successful treatment. While we focused on the regulation of drug resistance-associated miRNAs common between different cancers and drug classes, there are likely various miRNA that are specific to different drug treatments and cancers. However, the miRNA dysregulations discussed may have therapeutic value beyond AML. Furthermore, although we describe several drug resistance proteins, our analysis only focused on miRNA specifically implied in drug resistance where they were demonstrated to have direct activity and as such, the list is not exhaustive (190).

There are also many other molecular changes that occur in the development of drug resistance such as copy number variations, aberrant methylation, and aberrant post-transcriptional and post-translational processing (191, 192). The modulation of miRNAs offers a new perspective on drug resistance as miRNA replacement therapy and miRNA inhibition therapy raises the potential of developing new and effective drug therapies. Subtle miRNA changes can lead to significant changes in protein-coding gene expression and can consequently lead to changes in tumor progression and patient outcome. Experimental success *in vitro* and *in vivo* models may point to the likely coming of more miRNA-based clinical trials.

Previously, Mrx34 emerged as a promising therapy for the treatment of unresectable primary liver cancer. Due to multiple immune-related adverse events, this therapy was terminated in phase I although there was evidence of benefit in a subset of patients (193). Its promise came from being a p53-response element that was thought to mediate p53's antitumor effects and consequently affecting downstream signaling in proliferation arrest and induction of apoptosis by targeting *c-MYC, CDK6,* and *c-MET* (194). However, recent research now demonstrates that it may not always behave as a tumor-suppressor either and furthermore, p53 may also be a direct target of *miR-34a* (138, 195). In liver cancers with β-catenin mutations, it is demonstrated that LNA-34a, a *miR-34a* inhibitor, displays antitumor effects. This is suggested to occur through blocking HNF-4α targeting which in turn decreases cyclin D1 and inhibits proliferation (196, 197).

A *miR-16* mimic has also been recently introduced in patients in an open-label phase I clinical trial for mesothelioma and non-small cell lung cancer (NSLC). *miR-16* was shown to be dysregulated in many different cancers (87, 89, 90, 103–105, 124, 198). A directed analysis in mesothelioma showed that *miR-16* is reduced in patient samples and that a knock-in of a *miR-16* mimic is tumor suppressive (198). This observation was repeated in xenografted mice with high success (198). Currently, there are no miRNA-based therapies for drug resistant AML or AML-related diseases.

Currently, there are two miRNA-based therapies intended to treat different cancers that are on-going or with pending results. MesomiR-1, a *miR-16* mimic, was in a multi-center Phase I trial intended to treat mesothelioma and NSLC. This trial has been completed as of January 2017 and the results are currently pending. MRG-106 is a miRNA inhibitor that targets *miR-155* that is currently being examined in cutaneous T-cell lymphoma and mycosis fungoides. Like mesomiR-1, it is also currently in phase I. It is thought to block the action of *miR-155* from targeting tumor suppressors such as *C/EBP*β and altering the TGF-β response (199). This study is currently still recruiting patients. These studies may offer promise of miRNA treatment as therapy and pave the way for future studies similar in nature.

CONCLUDING STATEMENT

Today, the main hurdle for miRNA-based therapies remains to be the method of delivery. Many types of viruses are thought to be potentially useful for treatment and many stabilizing modifications such as phosphorothioate, methyl- and fluoro-substitutions on RNA species may help to overcome this hurdle (200, 201). Given the diverse set of roles that miRNAs play in regular cellular function, it is evident that clear elucidation of specific miRNA mechanisms may be required before their integration into modern cancer therapy (202). In contrast, due to the dependence and overexpression of a few coding mRNA in tumorigenic cells, it is possible that miRNAs may have a higher therapeutic index. miRNAs may prove to be an important addition to treatment in the years to come to treat drug resistant cancers in the future.

AUTHOR CONTRIBUTIONS

MG contributed to the research, figure design, and writing of manuscript. LS contributed to the research, editing, and overall design of manuscript.

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REVIEW
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New Challenges to Study Heterogeneity in Cancer Redox Metabolism

Rui Benfeitas[1], *Mathias Uhlen*[1], *Jens Nielsen*[1,2] *and Adil Mardinoglu*[1,2]*

[1] *Science for Life Laboratory, KTH Royal Institute of Technology, Stockholm, Sweden,* [2] *Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden*

Reactive oxygen species (ROS) are important pathophysiological molecules involved in vital cellular processes. They are extremely harmful at high concentrations because they promote the generation of radicals and the oxidation of lipids, proteins, and nucleic acids, which can result in apoptosis. An imbalance of ROS and a disturbance of redox homeostasis are now recognized as a hallmark of complex diseases. Considering that ROS levels are significantly increased in cancer cells due to mitochondrial dysfunction, ROS metabolism has been targeted for the development of efficient treatment strategies, and antioxidants are used as potential chemotherapeutic drugs. However, initial ROS-focused clinical trials in which antioxidants were supplemented to patients provided inconsistent results, i.e., improved treatment or increased malignancy. These different outcomes may result from the highly heterogeneous redox responses of tumors in different patients. Hence, population-based treatment strategies are unsuitable and patient-tailored therapeutic approaches are required for the effective treatment of patients. Moreover, due to the crosstalk between ROS, reducing equivalents [e.g., NAD(P)H] and central metabolism, which is heterogeneous in cancer, finding the best therapeutic target requires the consideration of system-wide approaches that are capable of capturing the complex alterations observed in all of the associated pathways. Systems biology and engineering approaches may be employed to overcome these challenges, together with tools developed in personalized medicine. However, ROS- and redox-based therapies have yet to be addressed by these methodologies in the context of disease treatment. Here, we review the role of ROS and their coupled redox partners in tumorigenesis. Specifically, we highlight some of the challenges in understanding the role of hydrogen peroxide (H_2O_2), one of the most important ROS in pathophysiology in the progression of cancer. We also discuss its interplay with antioxidant defenses, such as the coupled peroxiredoxin/thioredoxin and glutathione/glutathione peroxidase systems, and its reducing equivalent metabolism. Finally, we highlight the need for system-level and patient-tailored approaches to clarify the roles of these systems and identify therapeutic targets through the use of the tools developed in personalized medicine.

Keywords: cancer heterogeneity, redox biology, reactive oxygen species, systems biology, personalized medicine

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Miguel Ángel Medina,
University of Málaga, Spain

***Correspondence:**
Adil Mardinoglu
adilm@scilifelab.se

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INTRODUCTION

Redox metabolism is closely intertwined with cell physiology, and reactive oxygen species (ROS) are central players in health and disease. For instance, these oxygen-derived species are involved in cancer (Reuter et al., 2010), neurodegenerative diseases (Sultana et al., 2006), aging (Höhn et al., 2013), and diabetes (Evans et al., 2002). They are produced intracellularly by several processes and dedicated enzymes, such as NADPH oxidases (Nauseef, 2008) and in multiple cellular compartments (Messner and Imlay, 2002; Chen et al., 2008; Murphy, 2009; Brown and Borutaite, 2012). Due to their high membrane permeability (Chance et al., 1979), extracellularly produced ROS (Hampton et al., 1998; Babior et al., 2002) may quickly enter cells, or they may diffuse across compartments (Bienert et al., 2007; Marchissio et al., 2012). In many diseases, imbalances in ROS metabolism lead to oxidative stress. As result, cells face toxic outcomes of protein, lipid, and nucleic acid oxidation (Garrison, 1987; Cooke et al., 2003; Smith and Murphy, 2008; **Figure 1A**). For instance, DNA oxidation by ROS promotes mutagenesis, cancer initiation, and progression (Shibutani et al., 1991; Cooke et al., 2003; Sabharwal and Schumacker, 2014), and at high concentrations, ROS may cause cell apoptosis (Gao et al., 2013).

In turn, low ROS concentrations have important physiological roles (D'Autréaux and Toledano, 2007). They regulate cell-cycle progression (Havens et al., 2006), proliferation (Choe et al., 2012), growth (Arnold et al., 2001), and important signaling processes (Finkel, 2011; Rigoulet et al., 2011). For instance, hydrogen peroxide (H_2O_2) regulates the activity of kinases (Gotoh and Cooper, 1998; Paulsen et al., 2012), which control proliferation, differentiation, and apoptosis. NFE2L2 (also known as NRF2) responds to oxidative stress and regulates GSH biosynthesis and reduction, the expression of several proteins involved in antioxidant defense (glutathione peroxidases, transferases, peroxiredoxins, thioredoxins, and thioredoxin reductases), and NADPH production (Gorrini et al., 2013). Many of these redox-regulated processes are not directly controlled by ROS but rather by their redox partners. For instance, redox signaling transduction is often mediated by peroxiredoxins, thioredoxins, and other thiol-reacting proteins (Saitoh et al., 1998; Giannoni et al., 2005; Morinaka et al., 2011). These proteins ensure the high specificity required for efficient signaling transduction (Nagy and Winterbourn, 2010; Winterbourn, 2013; Marinho et al., 2014; Netto and Antunes, 2016). Together with other cellular antioxidants, such as catalases and dismutases, these redox systems prevent toxic ROS accumulation while permitting special-temporal selectivity and the maintenance of important redox signaling functions.

In cancer, mitochondrial dysfunction and metabolic changes promote constant oxidative stress (Szatrowski and Nathan, 1991; Hileman et al., 2004); however, this does not result in apoptosis. Cancer cells promote the expression of antioxidant defenses or reducing equivalents that enable their activity (Janssen et al., 1999; Miranda et al., 2000; Hileman et al., 2004), thus avoiding ROS-induced apoptosis and enabling proliferation, despite high mutagenesis (Toyokuni et al., 1995; Kondo et al., 1999) and metastasis (Ishikawa et al., 2008; **Figure 1B**). ROS closely interact with iron (Galaris et al., 2008) and central (Robbins et al., 2012; Hart et al., 2015; Miar et al., 2015) metabolism, and they are controlled by several transcription factors and tumor suppressors (Gao et al., 2007; Frohlich et al., 2008; Gupta et al., 2012; Gorrini et al., 2013; Hornsveld and Dansen, 2016). Additionally, antioxidant enzymes may display high or low expression in cancer cells (Ray et al., 2000; Oltra et al., 2001; Skrzydlewska et al., 2005; Glorieux et al., 2015), suppress tumorigenesis or promote metastasization (Zhao et al., 2001; Liu et al., 2012; Robbins et al., 2012; Miar et al., 2015), and display synergistic responses (Harris et al., 2015). As result, systematic approaches may capture these complex responses and provide insights into the mechanisms underlying the diverse phenotypic responses in cancer.

Systems biology presents promising approaches for capturing and studying complex cellular responses (Mardinoglu et al., 2013b; Ghaffari et al., 2015b). The application of such frameworks to clinical challenges is referred to as systems or network medicine (Mardinoglu and Nielsen, 2012, 2016). The complexity of biological pathways in cells and tissues may be captured through reconstruction of biological networks, including genome-scale metabolic models (GEMs), transcriptional regulatory networks, protein–protein interaction networks, and signaling networks, in an integrated approach that aims to understand entire cell processes at the systems level (Mardinoglu and Nielsen, 2015; Zhang et al., 2016). These networks may also be integrated with each other for a holistic understanding of the relationships between cellular networks, function, and disease (Bjornson et al., 2016; Lee et al., 2016; Mardinoglu and Uhlén, 2016). Generation of omics data for major human tissues enabled the generation of comprehensive biological networks (Kampf et al., 2014a; Lindskog et al., 2015; Uhlén et al., 2015, 2016; Thul et al., 2017), which have been successfully employed in revealing the underlying mechanisms involved in the occurrence of obesity (Mardinoglu et al., 2013a, 2014b, 2015a), type 2 diabetes (Väremo et al., 2015), non-alcoholic fatty liver disease (Kampf et al., 2014b; Mardinoglu et al., 2014a, in press; Hyötyläinen et al., 2016), and cancer (Agren et al., 2012, 2014; Weinstein et al., 2013; Zack et al., 2013; Leiserson et al., 2014; Yizhak et al., 2014b; Aran et al., 2015; Bjornson et al., 2015; Peng et al., 2015; Elsemman et al., 2016). Personalized models have also been used in the identification of potential therapeutic targets and biomarkers (Faratian et al., 2009; Agren et al., 2014; Bjornson et al., 2015; Mardinoglu et al., 2017; Nielsen, 2017). To date, small scale redox networks have also been analyzed (Zhang et al., 2010; Zhou et al., 2011; Zhan et al., 2012). However, despite extensive evidence highlighting the importance of ROS, antioxidants and other redox players in cancer, systems approaches have yet to systematically examine the role of redox metabolism in this disease and uncover potential personalized treatment strategies.

Here, we highlight some recent findings about important biological processes that are crucial in tumorigenesis: ROS, their redox partners, and reducing equivalents. We start by overviewing some of the main biochemical properties of ROS and their effectors. We then discuss the role of antioxidants and their reactions in tumorigenesis, focusing on thiols and reducing equivalents due to their importance

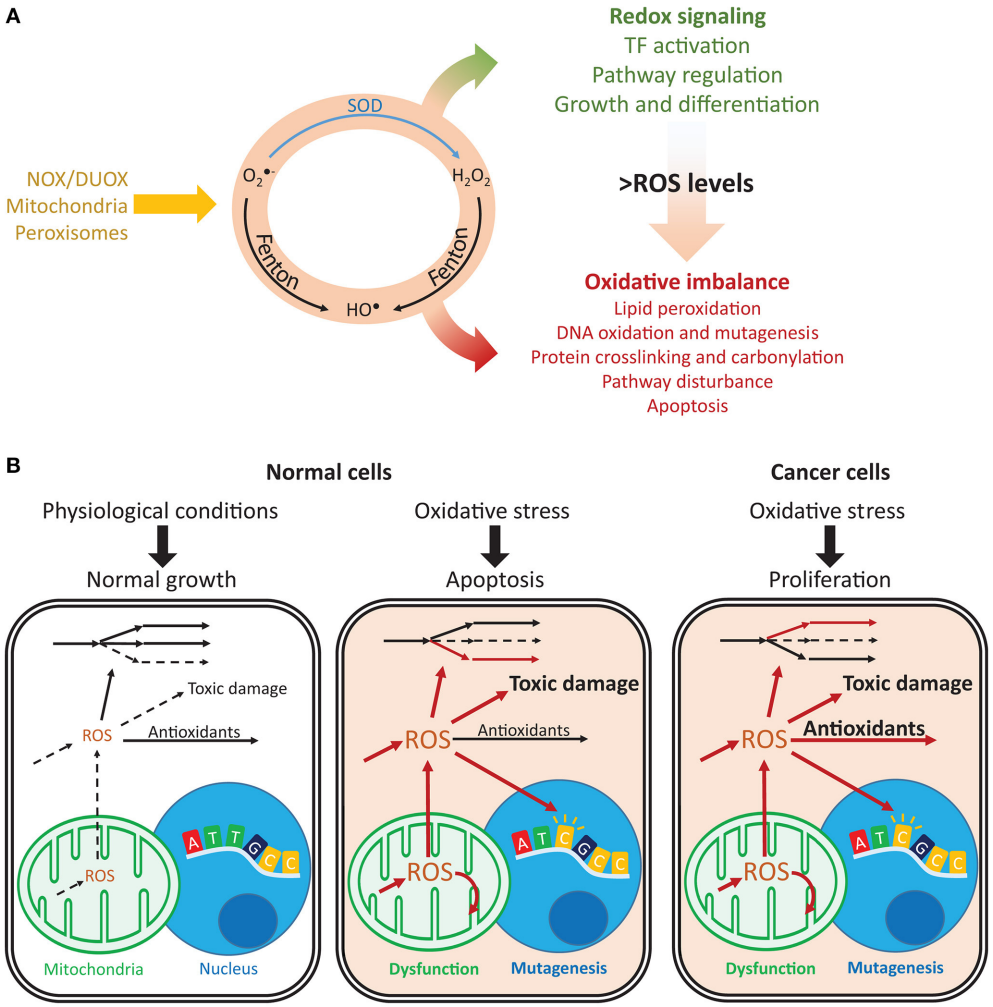

FIGURE 1 | Imbalances in ROS and redox cycles lead to contrasting outcomes in normal and cancer cells. **(A)** ROS are intracellularly produced by NADPH oxidases and dual oxidases (NOX/DUOX) through mitochondrial oxidative phosphorylation and in peroxisomes. Their interconversion (orange circle) occurs through enzyme catalyzed and non-catalyzed reactions. For instance, metal-catalyzed Fenton reactions produce $HO^{\bullet}$ from $O_2^{\bullet-}$ and H_2O_2. Under low ROS levels, these oxidants control important signaling reactions, activating transcription factors, regulating pathways and controlling cell growth and differentiation. Under high ROS levels, oxidation of lipids, nucleic acids, and proteins is toxic and may disturb pathways and lead to cell death. **(B)** Normal and cancer cells present important differences in their responses to oxidative stress. Under normal conditions, ROS production is low and antioxidant defenses are sufficient to prevent toxic damage. Under oxidative stress, the promoted production of ROS overcomes the cell's capacity for detoxification and results in increased toxic damage and pathway disruption, which may lead to mitochondrial dysfunction, mutagenesis and ultimately apoptosis. In turn, cancer cells are under constant oxidative stress, which through upregulation of antioxidant defenses, prevents apoptosis while maintaining ROS toxicity. Arrows indicate fluxes, increasing from dashed to continuous, in red. $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; $HO^{\bullet}$, hydroxyl radical; SOD, superoxide dismutase.

in ROS and redox homeostasis. Finally, given the high heterogeneity of redox responses and the intricate crosstalk between redox and central metabolism, we highlight how system-level and patient-tailored approaches may help to identify potential cancer targets and provide mechanistic insights into redox cancer responses. These discussions do not aim to be exhaustive descriptions of all biological processes and regulators of redox homeostasis, and the interested reader may find excellent reviews on these topics elsewhere (e.g., Gao et al., 2007; Frohlich et al., 2008; Gupta et al., 2012; Gorrini et al., 2013; Hornsveld and Dansen, 2016).

BIOCHEMISTRY OF ROS AND REDOX SYSTEMS

Molecular oxygen freely diffuses across cell membranes and promotes the formation of intracellular ROS through electron abstraction. ROS may be classified as radicals and non-radicals. Radicals have unpaired electrons and include superoxide ($O_2^{\bullet-}$) and the hydroxyl ($HO^{\bullet}$) radicals. Non-radical ROS do not have unpaired electrons, and they include H_2O_2. Here, we focus on these three ROS due to their patho-physiological importance. ROS metabolism yields many other less reactive, abundant,

or stable ROS and is highly intertwined with other important reactive species (such as Reactive Nitrogen Species, Weidinger and Kozlov, 2015).

ROS are formed in several intracellular compartments. Most notably, they are produced in peroxisomes through fatty acid oxidation (Fransen et al., 2012), in mitochondria during oxidative phosphorylation and in the cellular and intracellular membranes by NADPH oxidases (EC 1.6.3.1; Nauseef, 2008; Kowaltowski et al., 2009; Murphy, 2009; Brown and Borutaite, 2012; Fransen et al., 2012). Some crosstalk exists between these systems. For instance, mitochondrial-produced ROS promote $O_2^{\bullet-}$ generation by NADPH oxidases, which may have important functions during phagocytosis (Dikalov, 2011). It is currently unclear which of the compartments above contributes the most to intracellular ROS production, although mitochondria are often cited as the main cellular ROS source (Brown and Borutaite, 2012).

The reactions involving ROS and their cellular targets lead to the interconversion of various types of ROS (**Figure 2**). For instance, mitochondrial- and cytoplasmic-produced $O_2^{\bullet-}$ is dismutated to H_2O_2 by superoxide dismutases (SOD, EC 1.15.1.1). $O_2^{\bullet-}$ is fairly unreactive to most electron-rich centers due to its anionic charge, but it reacts with nitric oxide to form peroxynitrite (Huie and Padmaja, 1993) and oxidizes iron-sulfur clusters, thereby producing H_2O_2 and $HO^{\bullet}$ (Rouault and Klausner, 1996). These clusters are found in multiple intracellular compartments (Tong et al., 2000), and their oxidation by $O_2^{\bullet-}$ (or H_2O_2) leads to iron release and the inactivation of metabolically important enzymes, such as those involved in amino acid biosynthesis (Wallace et al., 2004) or carbohydrate metabolism (Gardner et al., 1995). It is currently unclear whether most $O_2^{\bullet-}$ is used toward nitric oxide metabolism, if it reacts with metal clusters, or if it is dismutated to H_2O_2. Both dismutases and nitric oxide react with $O_2^{\bullet-}$ with near diffusion-limited rate constants ($k > 10^9$ M^{-1} s^{-1}, Bannister et al., 1973; Huie and Padmaja, 1993), and $O_2^{\bullet-}$ is very reactive with some iron-sulfur cluster-bearing enzymes ($k \approx 10^6$–10^7 M^{-1} s^{-1}, Flint et al., 1993), but it is unreactive with amino acid residues (Bielski and Shiue, 1979). The fate of $O_2^{\bullet-}$ depends on the local availability of the other reactants or enzymes and likely varies between cells and under different conditions, although it is generally assumed that most $O_2^{\bullet-}$ is dismutated to H_2O_2 (Forman et al., 2010).

In turn, H_2O_2 reacts slowly with most biological compounds, such as free glutathione ($k < 10$ M^{-1} s^{-1}, Winterbourn and Metodiewa, 1999) and phosphatases ($k \approx$ 10–200 M^{-1} s^{-1}, LaButti et al., 2007; Marinho et al., 2014). However, it may display extremely high reactivities with selected protein thiols due to their neighboring chemical environment ($k \approx 10^5$–10^8 $M^{-1}s^{-1}$, Peskin et al., 2007; Trujillo et al., 2007; Manta et al., 2009). It is decomposed into water and molecular oxygen as a result of dismutation by catalases (EC 1.11.1.6), or it is reduced to water by peroxidases and peroxiredoxins (EC 1.11.1.15). Often, several of these mechanisms are present in the same cells. Protection against H_2O_2 is accomplished through glutathione peroxidase, catalase, and peroxiredoxin 2 in human erythrocytes (Johnson et al., 2005; Low et al., 2007; Benfeitas et al., 2014). These defenses are unlikely to be redundant in their functions: while catalase is an efficient H_2O_2 scavenger, even under high oxidative loads, peroxiredoxin 2 has limited reduction under such conditions (Low et al., 2007), resulting in a lower contribution for H_2O_2 consumption. However, peroxiredoxin 2 and its coupled cycles

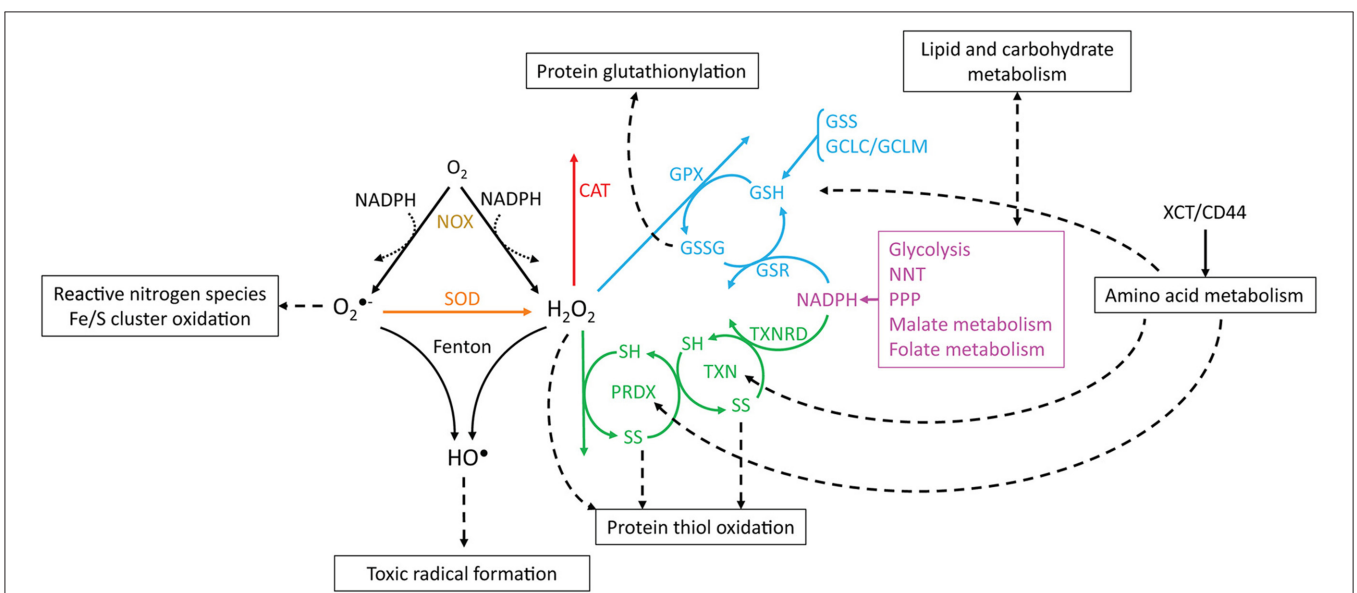

FIGURE 2 | Reactions involving ROS, antioxidant systems and energy metabolism. $O_2^{\bullet-}$ and H_2O_2 are produced from oxygen through reactions that may oxidize NADPH (dotted arrows, e.g., catalysis by NADPH oxidases). $O_2^{\bullet-}$ is dismutated to H_2O_2 by SOD, and $O_2^{\bullet-}$ and H_2O_2 may be converted to $HO^{\bullet}$ by Fenton reactions. CAT, PRDX and GPX scavenge H_2O_2. The catalytic cycles of PRDX and TXN are represented, where SH and SS, respectively, indicate reduced and oxidized (disulfide) thiols. Boxes and dashed arrows indicate the external processes with which the metabolites are associated. For example, XCT/CD44 mediates cysteine import, which may then be incorporated into proteins, such as TXN and PRDX, or may be metabolized to yield GSH. Colors indicate proteins or processes from the same pathway.

display desirable redox signaling properties (Benfeitas et al., 2014), which are further discussed below.

$O_2^{\bullet-}$ and H_2O_2 are also involved in the production of $HO^{\bullet}$ through iron-catalyzed Fenton reactions involving heme peroxidases or iron/sulfur clusters (Fenton, 1894; Chen and Schopfer, 1999; Koppenol, 2001). Iron accumulation and $HO^{\bullet}$ production have been extensively associated with carcinogenesis, and iron chelators have been employed as therapeutic drugs in cancer (reviewed by Torti and Torti, 2013; Bystrom and Rivella, 2015). Due to its electrophilic nature, $HO^{\bullet}$ preferably oxidizes electron-rich sites, reacting with nucleic acids, lipids, and proteins with diffusion-limited rate constants (Von Sonntag, 1987; Buxton et al., 1988; Stadtman and Levine, 2003; Sharma and Rokita, 2013). This promotes DNA strand breaks, lipid peroxidation and protein carbonylation, crosslinking, and cleavage. The products of these reactions are toxic, and they often promote radical propagation and damage to nearby molecules through subsequent chain reactions. Importantly, due to $HO^{\bullet}$'s very high and unselective reactions with biological compounds, no cellular antioxidants can feasibly scavenge this oxidant before it reacts with cellular contents. Instead, protection against the toxic outcomes of $HO^{\bullet}$ comes from preventing its formation by shielding iron from ROS or by scavenging H_2O_2 and $O_2^{\bullet-}$ before they yield $HO^{\bullet}$. The role of H_2O_2 in $HO^{\bullet}$ formation and consequential radical formation is also thought to be one of the main reasons behind H_2O_2's toxicity (Winterbourn, 1995).

As result of their different reactivities with biological compounds, the above ROS present varying stabilities and cellular roles. For instance, the fast and indiscriminate reactions of $HO^{\bullet}$ result in very small diffusion distances (≈80 Å, Roots and Okada, 1975) that are approximately the size of a small peptide. For this reason, $HO^{\bullet}$ is likely to oxidize molecules near its formation site (estimated half-life of 10^{-9} s, Pryor, 1986), and it is unfit to behave as a signaling molecule. $O_2^{\bullet-}$ is also regarded as a poor signaling effector because it does not permeate cell membranes and is quickly dismutated to H_2O_2, or it reacts with iron/sulfur clusters and nitric oxide. This results in low intracellular stability, hindering its diffusion across large distances. Therefore, while $O_2^{\bullet-}$ has some regulatory properties, these properties are possibly due to its role in nitric oxide and H_2O_2 metabolism (Brune, 2005; Thomas et al., 2006; D'Autréaux and Toledano, 2007; Kaewpila et al., 2008; Labunskyy and Gladyshev, 2013). In turn, H_2O_2 has emerged as the ROS that displays the best signaling properties. Its high stability and selective reactions with cellular compounds permit diffusion over distances of several micrometers (Winterbourn, 2008) and enable cell membrane crossing, which is also facilitated through specific channels (Bienert et al., 2007). H_2O_2 reacts with cellular thiols, including those contained in low molecular weight compounds, such as glutathione, and protein thiols, such as peroxiredoxins and thioredoxins (**Box 1** and **Figure 3**). These reactions convert an oxidizing equivalent into a redox signal, which may be transduced from protein to protein via thiol disulfide exchange or between glutathione and proteins, forming mixed disulfides. Together with intracellular thiols, H_2O_2 regulates the redox state and activity of several target proteins and has pivotal importance in both physiological and pathological conditions (D'Autréaux and Toledano, 2007). Due to this intricate association, any discussion about the involvement of ROS in tumorigenesis also needs to consider the role of thiols and other antioxidant

BOX 1 | Thiols as important redox signaling sensors and effectors.

Cysteine's thiol side chains (R-SH) are often very reactive with H_2O_2. They undergo a series of reversible or irreversible redox transitions, which are represented here through the catalytic cycles of a typical 2-Cys peroxiredoxin/thioredoxin (**Figure 3**). In this cycle, the reduced form is subsequently oxidized by H_2O_2 to sulfenic (R-SOH) and sulfinic (R-SO_2H) acids. The sulfinic form may be irreversibly oxidized to sulfonic (R-SO_3H) forms *in vitro*, but it is currently unclear whether this process occurs *in vivo*. Sulfinic acids may be reduced to sulfenic acids by specific proteins (e.g., sulfiredoxins, SRX) at the expense of ATP and the oxidation of TXN and GSH (Chang et al., 2004). Sulfenic acids may also conjugate to form intra- or inter-molecular disulfide bonds (R-SS-R'). Disulfides are then reduced at the expense of reducing equivalents, such as those found in NADPH (e.g., oxidized GSH or thioredoxin reduction by reductases), or by disulfide exchange with other proteins. Therefore, cysteine oxidation by H_2O_2 may be transduced to partner proteins or small molecular weight compounds.

Due to their chemical and kinetic properties, the systems above have potentially different signaling properties. Although they exhibit slow reactivities with H_2O_2 when isolated ($k \approx 2.9\ M^{-1}\ s^{-1}$ for free Cys, Winterbourn and Metodiewa, 1999), some cysteine thiols display extremely high reactivities ($k \approx 10^5$–$10^8\ M^{-1}\ s^{-1}$ for peroxiredoxins, Trujillo et al., 2007; Manta et al., 2009). GSH is very abundant, but it is relatively unreactive with H_2O_2 *per se*, so the kinetics of glutathione peroxidases should be considered when assessing glutathione's intracellular role in H_2O_2 detoxification and signaling. These differences in reactivity also manifest within the same pathway. Reduced and sulfenic forms quickly react with H_2O_2, unlike sulfinic and sulfonic acids, which are relatively unreactive with H_2O_2. The process of thiol oxidation to disulfide exchange may transduce oxidative equivalents to target proteins, as observed in the proteins above (Jarvis et al., 2012; Naticchia et al., 2013; Sobotta et al., 2015). For instance, thiol-disulfide exchange between peroxiredoxins and phosphatases/kinases is a mechanism for explaining H_2O_2-induced signaling regulation despite the low reactivity of H_2O_2 with phosphatases/kinases (Ray et al., 2012; Marinho et al., 2014; Sobotta et al., 2015; Latimer and Veal, 2016). Importantly, peroxiredoxin-mediated disulfide exchange controls the activity of several proteins involved in cancer (Park et al., 2007; Jarvis et al., 2012; Sobotta et al., 2015), which reinforces the role of H_2O_2 and redox metabolism in this disease. Similar to disulfide exchange, the oxidation of glutathione may lead to S-glutathionylation of proteins, which also regulates their activities (e.g., peroxiredoxin 2, Peskin et al., 2016). These and other properties possibly explain the involvement of PRDXs and TXNs in redox signaling, and they point toward these proteins as good redox sensors and signaling transducers (Benfeitas et al., 2014; Latimer and Veal, 2016; Netto and Antunes, 2016; Tomalin et al., 2016).

It should be noted that many of these redox systems depend on reducing equivalents to maintain their activity. Reducing equivalents are any molecules that act as electron donors in reactions, typically in reference to NADH and NADPH. These species are used by several enzymes, such as reductases, which couple their oxidation to the reduction of thioredoxin or glutathione. This provides another possible layer of selectivity in redox homeostasis: should NADPH utilization be prioritized toward one system over another, the physiological role of the former would also be prioritized over the latter. Interestingly, the link between energy metabolism and redox metabolism goes beyond NAD(P)H-enabling reductase activity. For instance, glyceraldehyde-3-phosphate dehydrogenase, an NADH-producing enzyme essential to glycolysis, is inactivated by glutathionylation and H_2O_2-induced disulfide formation (Little and O'brien, 1969; Mohr et al., 1999). ROS and redox-coupled processes thus not only consume reducing equivalents but also regulate energy metabolism.

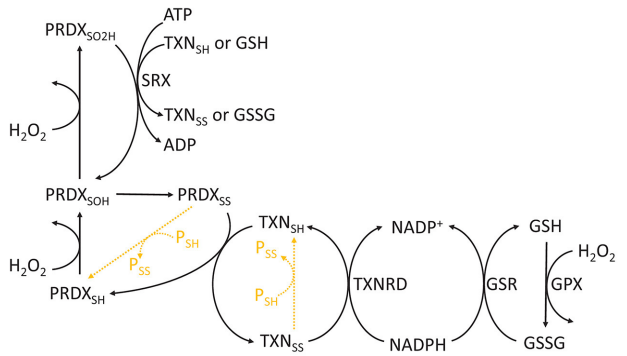

FIGURE 3 | Chemical cycle of PRDXs, TXNs, and GSH. The redox state of PRDXs and TXN is indicated as follows: SH, reduced cysteine thiol; SOH, sulfenic acid; SO_2H, sulfinic acid; SS, disulfide. Orange dashed reactions highlight disulfide exchange between peroxiredoxins and thioredoxins and other proteins.

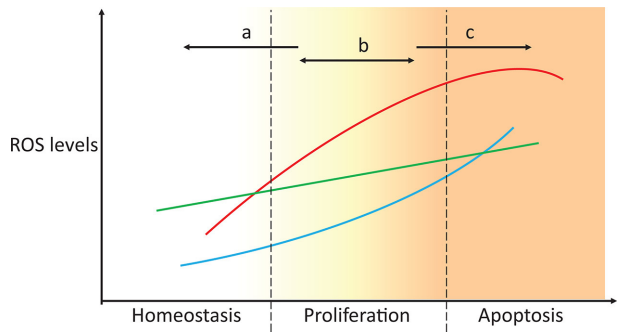

FIGURE 4 | Targeting ROS homeostasis as a strategy for changing cell fate. In proliferative conditions, such as cancer, targeting antioxidant systems could move the redox state of the cell to either promote normal redox homeostasis or apoptosis (strategies a and c, respectively). Unsuccessful tackling of antioxidant metabolism results in the cells maintaining a proliferative state, which potentially enhances malignancy. The three lines represent the high heterogeneity between individuals, tissues, and cancer types.

defenses. Although these systems have been widely studied in cancer, the role of ROS, antioxidant defenses, and redox signaling transducing partners in cancer is only now emerging beyond antioxidant activities. Further ROS-centered studies aimed at clarifying these properties and their involvement in cancer are still required.

ROS AS ONCOGENIC DRIVERS AND TARGETS OF THERAPEUTIC STRATEGIES

The role of ROS in cell physiology is highly dependent on their levels. Under physiological levels, ROS regulate a number of signaling processes by reacting with proteins, genes, and transcription factors. ROS control adaptation to hypoxia, regulation of differentiation, immunity, and longevity (Sena and Chandel, 2012). However, the accumulation of ROS beyond physiological levels promotes cell proliferation, angiogenesis, and even apoptosis (D'Autréaux and Toledano, 2007; Cairns et al., 2011; **Figure 4**), and ROS also control cell-cycle progression (Menon and Goswami, 2007). Oncogene-induced senescence promotes AMP-activated protein kinase activation, mitochondrial dysfunction and ROS production, which trigger senescence, thereby forming a positive feedback loop. Cancer cells display high ROS production (Szatrowski and Nathan, 1991; Ray et al., 2000), which is often also associated with antioxidant imbalances (Skrzydlewska et al., 2005). This results in damage to nuclear (Shibutani et al., 1991) and mitochondrial DNA (Ishikawa et al., 2008; Weinberg et al., 2010). Mutations in nucleic acids may be particularly toxic for the cell if they occur in tumor suppressors or oncogenes. DNA mutations (Higinbotham et al., 1992; Du et al., 1994), in turn, promote ROS generation, thereby resulting in a vicious cycle of ROS production and mutagenesis concomitant with high proliferation. Mitochondrial-generated ROS are also essential for tumor aggressiveness and metastasis (Ishikawa et al., 2008; Weinberg et al., 2010; Goh et al., 2011), and increased cytoplasmic and mitochondrial ROS levels are observed in metastatic nodules and circulating tumors when compared to subcutaneous tumors (Piskounova et al., 2015).

ROS also stabilize the factors that drive tumor initiation and progression (Gao et al., 2007), and promote protein oxidation and the formation of toxic protein carbonyls (Stadtman and Levine, 2003). Protein carbonylation is an irreversible process present in cancer cells (Thanan et al., 2012). Carbonyls may propagate to other proteins or lipids, which may result in the formation of toxic byproducts through chain reactions. ROS-induced lipid peroxidation products, such as 4-hydroxy-2-nonenals, also have multiple physiological roles under low levels, but they become toxic upon accumulation. These species also accumulate in cancer cells (Skrzydlewska et al., 2005; Ayala et al., 2014; Zhong and Yin, 2015).

Due to the toxic effects of ROS accumulation in cancer cells and the fact that some ROS responses may be exclusive to cancer cells but not to their healthy counterparts (Hileman et al., 2004), antioxidants were envisioned as potentially important drug targets in cancer treatment. Initial ROS-focused clinical trials aimed to prevent ROS accumulation (**Figure 4**, strategy a). In the Linxian study (Blot et al., 1993) stomach cancer patients supplemented with selenium, vitamin E and β-carotene exhibited lower mortality. However, antioxidant-supplemented diets often failed to yield significant changes in cancer development, and in some cases, these diets even promoted tumorigenesis and metastasis (Omenn et al., 1996; Goodman et al., 2011; Klein et al., 2011; Sayin et al., 2014). This is because cancer cells often cope with increased ROS production by increasing the levels of antioxidant defenses or reducing equivalents that maintain their activity (Weinberg et al., 2010; DeNicola et al., 2011). This effect is also observed in metastases, where metastatic melanoma nodules are exposed to additional oxidative stress that is not observed in established subcutaneous tumors, and the nodules cope with this stress by promoting the expression of multiple NADPH-producing pathways (Piskounova et al., 2015). High antioxidant activities enable fast ROS-driven proliferation and metastasization, but the increased oxidative stress is insufficient to lead to apoptosis (**Figure 4**, strategy b). Higher antioxidant expression is also associated with the radioresistance observed

in certain cancer stem cell populations (Diehn et al., 2009). In turn, recent antioxidant-targeted therapeutic strategies have shifted their focus in the opposite direction, exploiting ROS toxicity as a means leading to cancer cell apoptosis (**Figure 4**, strategy c). These drugs often mimic ROS-generating enzymes (e.g., NADPH oxidases and superoxide dismutases), inhibit antioxidant enzymes (e.g., catalase), deplete thiol pools, such as GSH, or shift redox buffer ratios (e.g., GSSG/2 GSH), thereby promoting a more oxidizing intracellular state and cell apoptosis. These strategies have been discussed in great detail in recent reviews (Ushio-Fukai and Nakamura, 2008; Gupta et al., 2012; Gorrini et al., 2013; Tong et al., 2015).

The targets of many of these drugs are not completely understood, nor is it known whether the drugs are targeting the best redox effectors. Are the targeted compounds selective in treating cancer, but not healthy cells? Are they the best target in that pathway? Which combined drug treatments could improve treatment? These questions stem from an insufficient understanding of redox metabolism in tumorigenesis, which is greatly caused by the high variability in metabolic and redox responses. For instance, different antioxidant defenses that target the same ROS are up- and down-regulated in cancer cells (Skrzydlewska et al., 2005), complicating the interpretation of their role and that of their target ROS in cancer progression. This high variability is even observed between individuals with the same cancer type (**Figure 5**). Furthermore, genes involved in the same processes are differentially expressed. For instance, a high dispersion in gene expression levels is observed within peroxiredoxins (*PRDX1-6*), thioredoxins (*TXN* and *TXN2*), and thioredoxin reductases (*TXNRD1-3*), which are highly conserved protein systems involved in H_2O_2 scavenging and redox signaling transduction. As result of this variability, redox-focused therapeutic strategies (**Figure 4**) must consider patient-specific data to determine the best approach.

ROLE OF ANTIOXIDANT DEFENSES IN CANCER

Imbalances in antioxidant defenses are one of the hallmarks of cancer (Oberley and Oberley, 1997; Huang et al., 2000; Chung-man Ho et al., 2001; Hu et al., 2005; Murawaki et al., 2008). For instance, increased expression of *SOD* has been observed in multiple cancers (Ray et al., 2000; Skrzydlewska et al., 2005; Holley et al., 2012; Miar et al., 2015) and metastatic tissues (Miar et al., 2015). Its overexpression promotes carcinogenesis (Lu et al., 1997) and aggressiveness (Hempel et al., 2011). In turn, *SOD* deficiency is associated with a higher cancer incidence and DNA damage in mice (Van Remmen et al., 2003; Elchuri et al., 2005), and it has also been observed in some cancers (Oltra et al., 2001). *SOD* levels fluctuate throughout the cell cycle and regulate growth factor cancer signaling (Nelson et al., 2003; Juarez et al., 2008), cell cycle progression, and the energetic changes of cells upon cancer transformation (Hempel et al., 2011; Sarsour et al., 2014). Catalases, often regarded as the main cellular defenses against H_2O_2 in human cells, may be up- or down-regulated in cancer cells (Ray et al., 2000; Oltra et al., 2001; Skrzydlewska et al., 2005; Glorieux et al., 2015). Catalase treatment of highly metastatic cancer cell lines decreases migration and invasion (Liu et al., 2012). Due to their antioxidant

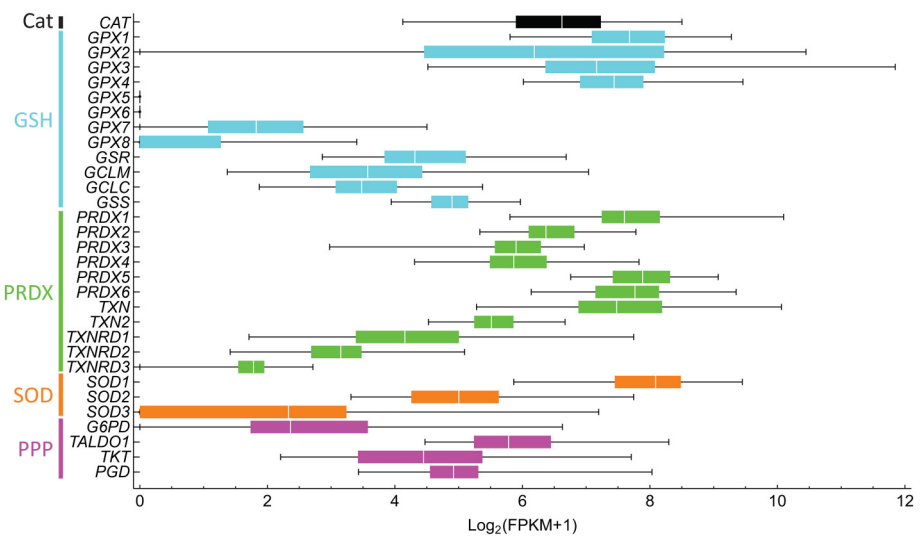

FIGURE 5 | Antioxidant gene expression greatly varies between liver hepatocellular carcinoma in different subjects. The gene expression of 50 subjects was downloaded from NCI's Genomic Data Commons, and fragments per kilobase transcript per million (FPKM) were computed. FPKM-values lower than one were considered to be non-expressed and were assigned a value of 0. The Log_2(FPKM + 1) were then computed. Bars are colored according to processes of the same pathway, as indicated on the left. Genes and respective proteins: CAT, catalase; GPX1-8, glutathione peroxidase; GSR, glutathione reductase; GCLM and GCLC, glutamate-cysteine ligase modifier and catalytic subunits, respectively; GSS, glutathione synthetase; PRDX1-6, peroxiredoxin; TXN and TXN2, thioredoxin; TXNRD1-3, thioredoxin reductase; SOD, superoxide dismutase; G6PD, glucose-6-phosphate dehydrogenase; TALDO1, transaldolase 1, TKT, transketolase, and PGD, 6-phosphogluconate dehydrogenase.

activities and correlation with decreased aggressiveness in certain tumors, catalases have been envisaged as potentially important therapeutic agents (Glorieux et al., 2011; de Oliveira et al., 2016). For instance, a recent study shows that catalase activity correlates well with the ability of pancreatic cancers to resist chemotherapeutic H_2O_2 treatment with ascorbate (Doskey et al., 2016). In turn, altered levels of glutathione peroxidases are amply reported in cancer (Lu et al., 1997; Skrzydlewska et al., 2005), and the reduction potential of glutathione is associated with the proliferative and apoptotic state of a cell (Buettner et al., 2013). For instance, several glutathione peroxidases are upregulated in hepatocellular cancer (Carlson et al., 2012), and GPX4-deficient mice die shortly after birth (Carlson et al., 2016). GPXs are responsible not only for reducing H_2O_2 but also for reducing other ROS, such as lipid peroxides (Thomas et al., 1990; Esworthy et al., 1993). Peroxiredoxins have also been associated with tumorigenesis. PRDX1-4 and 6 display significantly altered levels in some prostate cancers (Basu et al., 2011; Whitaker et al., 2013). PRDXs may act as tumor suppressors (Egler et al., 2005), and their increased gene expression is associated with metastasis and aggressiveness (Park et al., 2006; Chang et al., 2007; Stresing et al., 2012). For instance, PRDX2 is highly expressed in lung metastases, and its knockdown decreases the formation of lung metastasis (Stresing et al., 2012). PRDX2 is also highly expressed in breast carcinoma, which correlates with the formation of lung metastases. PRDXs are often upregulated in cancers, and they contribute to cancer survival and resistance to oxidative stress (Lu et al., 2014) and radiotherapy (Wang et al., 2005). Interestingly, knockouts of the *PRDX* genes do not always result in a favorable outcome. For instance, *PRDX1* knockout mice show premature death, increased DNA oxidation and increased tumorigenesis and malignancy, and *PRDX3* knockout results in increased protein carbonylation in adipose tissues (see Cao et al., 2009; Perkins et al., 2014 and references therein). These observations show the crucial role of antioxidant enzymes in cancerogenesis and survivability.

Alterations in antioxidant enzymes and ROS levels occur throughout cancer progression. Overexpression of mitochondrial *SOD2* acts as a tumor suppressor in skin and breast cancers (Zhao et al., 2001; Robbins et al., 2012), suggesting that high SOD activity promotes tumor initiation. However, SOD levels also increase with tumor progression in these and other cancers (Ray et al., 2000; Chung-man Ho et al., 2001; Dhar et al., 2011; Miar et al., 2015), with similar changes occurring in other antioxidants, such as cytoplasmic SOD1 and catalase (Miar et al., 2015). Similarly, metastases show increased SOD2 protein levels when compared with matched tissues, less metastatic cell lines, or primary tumors (Liu et al., 2012; Miar et al., 2015). These changes are often accompanied by directly proportional changes in intracellular concentrations of H_2O_2, but they are not always followed by changes in other antioxidants. Changes in ratios of antioxidant proteins (e.g., SOD/catalase and SOD/GPX1) translate into differential intracellular concentrations of H_2O_2, and they vary with cancer stage and between metastatic and primary tumor cells (Miar et al., 2015). These observations show that while a comparison of the antioxidant expression levels between cancer and matched tissues is informative, such a comparison has to consider the developmental stage of the cancer.

Importantly, these antioxidant defenses are not found in all cell compartments, which should also differentially affect the responses of cancer cells to therapeutic targeting. For instance, mitochondria rely on superoxide dismutase 2, peroxiredoxin 3, thioredoxin 2, and thioredoxin reductase 2 for antioxidant defense and signaling transduction, but they do not rely on catalase (Rabilloud et al., 2001; Jones, 2006). Considering the crucial role that mitochondrial-generated ROS have in cancer initiation, progression, and apoptosis, the targeting of key antioxidant and redox signaling transduction systems in this compartment (e.g., PRDX3 Li and Yu, 2015) may have important consequences for global cell metabolism. However, it should be noted that targeting these antioxidants may lead to compensatory responses by the other antioxidants. For instance, knockdown of *PRDX3* promotes the upregulation of other peroxiredoxins, including those located in the cytoplasm (*PRDX1-2* and *PRDX6*; Li et al., 2008, 2013; Goncalves et al., 2012). This is particularly important because these proteins may catalyze similar reactions in H_2O_2 scavenging and disulfide exchange, albeit with different mechanisms and specificities (Perkins et al., 2014).

In addition, cancer cells cope with increased intracellular ROS by promoting the synthesis of compounds that enable their activity (e.g., glutathione for GPXs, Buettner et al., 2013 and TXNs for PRDXs, Arnér and Holmgren, 2006; Kaimul et al., 2007). GSSG/GSH ratios are higher in many cancers (Oltra et al., 2001; Skrzydlewska et al., 2005), in metastatic nodules and in circulating tumor cells (Piskounova et al., 2015), and the enzymes involved in GSH recycling or synthesis are often upregulated (Carlson et al., 2012; Harris et al., 2015; Lien et al., 2016) as response to oxidative stress. Mutations in PI(3)K/Akt, which are common in some cancer types, such as breast cancer, stabilize and activate NFE2L2, thereby promoting the upregulation of enzymes involved in synthesizing or reducing GSH (glutathione synthetase GSS, and glutathione reductase GSR; Lien et al., 2016). Upregulation of GSS and GSR is also associated with the increased resistance to oxidative stress observed in breast cancer. The inhibition of GSH biosynthesis sensitizes cancer cells to H_2O_2 and is potentiated by the utilization of other antioxidant inhibitors (Lien et al., 2016). High intracellular GSH concentrations also block drug-induced cytotoxicity in myeloma cells (Starheim et al., 2016). Cumulative evidence thus points toward GSH biosynthesis and homeostasis as a therapeutic target. However, in some cancers, inhibition of the GSH pathway alone does not prevent tumor progression. In addition to GSS, GSH is also synthesized by glutamate-cysteine ligase (GCL), an enzyme that consists of a heavy catalytic (GCLC) subunit and a light regulatory (modifier, GCLM) subunit. In mouse models of breast cancer, GCLM-deficiency or GLCM inhibition by Buthionine-[S,R]-sulfoximine (BSO) significantly prevented cancer initiation. However, this effect occurs only before tumor onset, and BSO treatment after onset does not alter tumor burden due to a compensatory role of TXN (Harris et al., 2015). GSH and TXN both serve as substrates for proteins with redox-important roles, including glutathione peroxidases, peroxiredoxins, glutathione

and thioredoxin reductases, glutaredoxins, and sulfiredoxins (Björnstedt et al., 1994; Sun et al., 2001; Chang et al., 2004; Johansson et al., 2004; Peskin et al., 2016). Several cancers display increased expression of *TXN* and thioredoxin reductase 1 (*TXNRD1*) to compensate for GSH deficiency in $GCLM^{-/-}$ cells (Mandal et al., 2010; Harris et al., 2015). The inverse is also observed, where *TXNRD1*-deficiency promotes the expression of *GSR* and *GCLC*, but not of *PRDX1*, *CAT*, or *SOD1*, in liver cancer cells (Carlson et al., 2012). Given that GSH and thioredoxins are relatively unreactive with ROS *per se* (Chae et al., 1994; Winterbourn and Metodiewa, 1999), the promotion of GSH or TXN biosynthesis is possibly promoting the peroxidase activities of GPX and PRDX.

GLCM-deficient cells also present increased expression of the cystine transporters and stabilizers *XCT* and *CD44* (Lu et al., 2015). This increased import is used toward promoting cysteine biosynthesis, which, in turn, is used toward TXN biosynthesis. Other observations show that chemotherapy treatment promotes *XCT* and *GCLM* expression with a concomitant increase in GSH biosynthesis in a HIF1-dependent mechanism related to therapeutic resistance (Lu et al., 2015). Targeting XCT, GCLM, and other pluripotency-involved transcription factors impaired malignant transformation. It is currently unclear whether other cysteine-based antioxidants, such as peroxiredoxins, also benefit from increased cystine import. Importantly, it was also observed that TXNs and thioredoxin reductases are upregulated and co-localize in several cancers, particularly in more aggressive cancers (Soini et al., 2001; Lincoln et al., 2003). These enzymes are associated with tumor initiation (Shen et al., 2016), and they correlated with worse prognosis (Cadenas et al., 2010). *TXNRD1* knockdown significantly slowed tumor progression and metastasis in lung carcinomas (Yoo et al., 2006), but it promoted cancer incidence in liver (Carlson et al., 2012).

Due to their chemical similarities and synergistic properties, recent approaches have simultaneously targeted both TXN and GSH metabolism, and they are significantly more effective at reducing tumor volumes than when they are applied individually (Harris et al., 2015). Multiple other studies have shown this efficacy in combination with common cancer drugs or radiotherapy for multiple cancers (e.g., Lu et al., 2007; Sobhakumari et al., 2012; Rodman et al., 2016; Roh et al., 2016; Tanaka et al., 2016). Together, the intracellular redox states expressed in terms of reducing equivalents and thiol compounds (**Box 1**) not only influence several signaling processes but also control the reactivities of ROS and their redox partners by regulating ROS homeostasis. It is currently unclear whether the involvement of some of these antioxidant defenses in cancer is related to their detoxification role, redox signaling properties or both. For instance, PRDX2 has increased levels in some cancer cells, which correlates with lower cytoplasmic H_2O_2 concentrations and cellular resistance to oxidative stress (Stresing et al., 2012). PRDX2 displays peroxidase activity and is also involved in redox signaling transduction (Neumann and Fang, 2007), such as the positive regulation of JNK-dependent DNA repair (Lee et al., 2011). Since these H_2O_2-scavenging and redox signaling properties stem from the kinetic properties of PRDX2/TXN/TXNRD1, GPX1/GSH/GSR, and catalase systems (Benfeitas et al., 2014; Tomalin et al., 2016), further studies are required to understand whether the culprit of PRDX2's tumorigenic association is its role as a peroxidase, chaperone, or redox signaling transducer. It is unclear whether the alterations in GSH/TXN biosynthesis and PRDX/GPX levels are more important toward controlling redox signaling, detoxification, or both. For instance, some observations indicate that sulfiredoxins and peroxiredoxins promote tumor growth and metastasis by modulating phosphokinase signaling cascades (Wei et al., 2011). Do these proteins directly interact with their targets? PTEN binds to PRDX1, but not to PRDX2, and this promotes Akt-mediated proliferation (Cao et al., 2009). The PRDX1-PTEN complex dissociates upon H_2O_2-mediated oxidation. A localized accumulation of H_2O_2, such as that occurring near cell membranes or near ROS sources, would thereby alter PTEN-mediated signaling transduction and proliferation by direct PRDX1 oxidation (Woo et al., 2010) or by relaying a redox signal from another more abundant, H_2O_2-reactive PRDX. PRDX2 is a good candidate as a H_2O_2 sensor due to its high reactivity with H_2O_2 ($k \approx 10^7$–10^8 M^{-1} s^{-1}), and it was recently observed to transmit oxidative equivalents to the transcription factor STAT3 (Sobotta et al., 2015), thereby controlling tumor proliferation and survival (Yu et al., 2014). While disulfide exchange between PRDX1 and 2 remains to be shown, the above observations indicate that the direct reactions of peroxiredoxins with transcription factors are important proliferative processes controlled by H_2O_2. It also remains to be seen whether the promotion of TXN biosynthesis (Harris et al., 2015), which is often linked to added ROS protection, is instead enabling secondary signaling transduction reactions, and whether multiple PRDX isoforms act synergistically in this process. Studying cancer ROS metabolism should therefore consider the toxicity of these oxidants and signaling disruption.

Overall, the observations above highlight important features that should be considered in cancer studies. First, targeting one antioxidant defense may elicit compensatory behaviors by other antioxidant defenses. Second, the close relationship between antioxidant proteins (e.g., GPX and PRDX) and their redox partners (e.g., GSH and TXN) requires that the choice of suitable therapeutic targets considers possible synergisms between them. Third, the high variability in responses, even for the same tumor, requires that cancer treatment is designed in a case- and stage-specific manner, rather than a cancer-type approach. Finally, all of these considerations need to be considered to identify antioxidant pathways that are differentially regulated by cancer, but not by normal cells. The targeting of antioxidant defenses as an approach for cancer treatment should therefore require tissue- and subject-specific phenotypic characterization.

ENERGETIC CHANGES ARE COUPLED WITH MAINTENANCE OF THE ANTIOXIDANT ACTIVITY IN CANCER

Cancer cells display increased glycolytic activity and lower mitochondrial oxidative phosphorylation. Glucose uptake by breast, liver, colorectal, lung, and pancreatic cancers may reach 8–15 times the fluxes observed by surrounding normal

tissues (see Boros et al., 1998 and references therein). This metabolic shift, characterized by increased ATP production from glycolytic pathways rather than respiratory pathways, even under aerobiosis, is one of the most well-known metabolic hallmarks of cancer cells, and it is generally referred to as the Warburg effect (Warburg, 1956). This process is crucial to maintaining the high energetic demand of fast proliferative cells. However, the energetic changes extend beyond the Warburg effect and are intimately related to the redox responses of cancer cells. This is the case of the metabolic changes that alter NADPH production. For instance, the MYC-controlled expression of pyruvate kinase type M2 (*PKM2*) is higher in cancer cells and promotes the diversion of carbohydrate metabolism from glycolytic pathways to other pathways (Vander Heiden et al., 2009), including the pentose phosphate pathway (PPP). Carbohydrates are thus diverted from ATP production to generate reducing equivalents and building blocks, such as phosphopentoses and ribonucleotides, supporting the fast proliferation of cancer cells (Boros et al., 1997; Raïs et al., 1999). Because ROS also regulate carbohydrate metabolism (Robbins et al., 2012; Hart et al., 2015; Miar et al., 2015) and some enzymes couple redox metabolism and ATP phosphorylation (Chang et al., 2004), the crosstalk between energetic and redox metabolism extends beyond enabling NADPH-driven peroxidase antioxidant activities.

Many of the glycolytic and PPP enzymes that are involved in NADPH production are elevated in cancer cells. Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), which are both enzymes of the oxidative branch of PPP, catalyze the production of NADPH from hexoses entering the PPP from either glycolysis or the non-oxidative PPP. G6PD's activity is promoted by multiple oncogenic pathways upregulated in cancer (Stanton et al., 1991; Tian et al., 1994; Au et al., 2000; Wang et al., 2012; Zhang et al., 2014), and multiple studies have proposed that G6PD has pro-oncogenic activities (Wang et al., 2012; Patra and Hay, 2014; Zhang et al., 2014). *G6PD* overexpression leads to higher levels of intracellular NADPH, GSH, and nucleotide precursors, increased health span, and lower nucleotide oxidation (Nóbrega-pereira et al., 2016). These observations provide a clear link between PPP-mediated NADPH production and oxidative stress. In turn, G6PD deficiency severely limits cell resistance to oxidative stress (Pandolfi et al., 1995) and promotes oxidative damage to DNA (Jeng et al., 2013). These observations raise the hypothesis that a targeted inhibition of G6PD may be conducive to oxidative imbalance and ROS-mediated cell death. Considering that G6PD catalyzes the first and rate-limiting step of PPP and that it has a role in controlling the intracellular redox environment, this enzyme has been envisaged as one of the potentially most important therapeutic cancer redox targets (Wang et al., 2012; Patra and Hay, 2014; Zhang et al., 2014; Nóbrega-pereira et al., 2016), and it has been included in pre-clinical trials (Budihardjo et al., 1998; De Preter et al., 2015). *6PGD* is also upregulated in many cancers, including thyroid (Giusti et al., 2008), lung (Sukhatme and Chan, 2012), and cervical (Jonas et al., 1992) tumors. This enzyme is important for proliferation and tumor growth (Sukhatme and Chan, 2012; Shan et al., 2014), and its inhibition promotes senescence in lung cancer (Sukhatme and Chan, 2012). This phenotype results from altered glucose levels, but not altered NADPH levels (Sukhatme and Chan, 2012; Lin et al., 2015), suggesting that NADPH metabolism, and ultimately glucose metabolism, may adapt in such a way that compensates for the selective targeting of the PPP's enzymes. Interestingly, 6PGD suppression limits lipid biosynthesis and elevates intracellular ROS levels, and this effect translates into decreased tumor growth (Lin et al., 2015). However, conflicting observations regarding its importance in cancerogenesis (Sukhatme and Chan, 2012; Lin et al., 2015) also suggest that its role may vary depending on tissue and oncogenic background (Lin et al., 2015).

Other enzymes also promote downstream NADPH production. For instance, transketolase (TKT) and transaldolase (TALDO) are both enzymes of the non-oxidative PPP. While neither of them catalyze NADPH production, they are both important in directing the phosphorylated pentoses generated in the PPP back to glycolysis. Both enzymes are upregulated in cancer (Heinrich et al., 1976; Liu et al., 2010). TKT is required for cancer growth and controls resistance to oxidative stress by modulating NADPH levels. Its inhibition leads to higher intracellular ROS and decreased NADPH/$NADP^+$ ratios (Xu et al., 2016), and it sensitizes cells to drug treatment. Importantly, *TKT* knockdown increases oxidative PPP fluxes, but it also leads to lower NADPH levels, which is a striking observation considering that NADPH is produced through the oxidative PPP; this point remains to be clarified. *TALDO*'s expression is linked to metastasis in hepatocellular carcinoma (Wang et al., 2011). TALDO deficiency also elicits hepatocellular carcinoma and promotes the formation of malignant tumors (Hanczko et al., 2009). These outcomes are associated with redox imbalances (lower NADPH and GSH levels) due to the insufficient recycling of PPP metabolites to support NADPH production, and they are reverted with dietary supplementation of antioxidants. This insufficient recycling exposes the liver to added oxidative stress and decreases lifespan. Other NADPH-producing enzymes are also upregulated in cancer cells, and some of the isoforms are exclusive to proliferating cells (Mazurek et al., 2005), suggesting that these enzymes may be selective therapeutic targets. Altered glycolytic and PPP metabolism has been proposed for potential therapeutic targeting in cancer (Wang et al., 2012; Patra and Hay, 2014; Zhang et al., 2014; Wen et al., 2015; Hay, 2016). Importantly, the crucial role of the PPP in cancer development seems to be associated with its redox homeostasis properties rather than its production of ribonucleotide precursors, as observed in hepatocellular carcinoma (Xu et al., 2016). Further studies are required to understand whether similar observations are present in other cancers.

While the oxidative PPP represents the main source of cytoplasmic NADPH in proliferating cells (Fan et al., 2014), other sources contribute significantly. Serine (Mehrmohamadi et al., 2014), folate (Tedeschi et al., 2013; Fan et al., 2014; Piskounova et al., 2015), and malate (Jiang et al., 2013) pathways also produce and regenerate NADPH and have crucial roles in maintaining the redox status and buffering oxidative stress in cancer cells. A system-wide comparison the contributions of these pathways to NADPH production and ROS metabolism in cancer is beginning

to emerge (e.g., Tedeschi et al., 2013; Mehrmohamadi et al., 2014). In the context of antioxidant defense, most of these studies have focused on GSH-mediated ROS protection due to the close relationship between the PPP, serine/glycine metabolism, and *de novo* GSH biosynthesis. However, an analysis addressing the role of these pathways in supporting the activities of other important antioxidant defenses and redox signaling processes is currently missing. This becomes an important issue because defenses have different reducing equivalent requirements for activity, which also reflect their different antioxidant capacities and redox signaling roles. For instance, while antioxidant systems like PRDX/TXN/TXNRD and GPX/GSH/GSR stoichiometrically couple ROS detoxification to NADPH consumption, others, such as catalase, scavenge ROS while oxidizing virtually no NADPH. In normal cells, where energetic metabolism is limited, NADPH must be utilized toward lipid and cholesterol biosynthesis, and ROS decomposition by NADPH-consuming systems is thus an energetically expensive process. In cancer cells, where NADPH-producing fluxes are promoted, NADPH may be sufficiently abundant for cells to afford utilizing PRDX- or GPX-mediated detoxification. Importantly, the differences in the energetic requirements and kinetics of the PRDX/TXN/TXNRD, GPX/GSH/GSR, and catalase systems become particularly important if cancer treatments are targeting ROS metabolism by inhibiting NADPH production. ROS defenses may then be maintained by catalase or other energetically inexpensive processes, which is similar to what is observed in non-cancerous cells (Johnson et al., 2005; Benfeitas et al., 2014). Nevertheless, the lower cancer progression and increased ROS levels induced by the inhibition of NADPH-producing pathways indicate that this is a viable cancer therapy. A further understanding of the energetic requirements of antioxidant defense (e.g., PRDX/TXN vs. GSH/GPX pathways) may permit an efficient combination of anti-oxidant- and energetic-focused drug utilization for effective cancer treatment. Importantly, the heterogeneous gene expression and synergistic responses that may occur between alternative metabolic pathways at different cancer stages require an assessment of possible targets that considers specific oncogenic backgrounds.

SYSTEMS BIOLOGY AND PERSONALIZED MEDICINE APPROACHES ARE FUNDAMENTAL TO REVEALING REDOX RESPONSE IN CANCER

The observations above highlight the extensive crosstalk within and between ROS detoxification, redox signaling transduction, energy metabolism, and central metabolism. As such, the therapeutic targeting of cancer is more effectively strategized by addressing multi-pathway dysregulation (Pawson and Linding, 2008). Thus, while targeting the activity of specific enzymes may yield promising results *in vitro* and to a certain extent, *in vivo*, methods that encompass global metabolism are required to devise viable, cancer-specific treatment targets. Furthermore, these redox responses are highly heterogeneous, as has been observed by the different redox responses displayed by different cancer types, between individuals with the same cancer type, and between different cancer stages. Finding the best targets (**Figure 4**) and elucidating the mechanisms behind cancer phenotypes hence requires integrative analysis of a large number of biological networks, together with tissue- and patient-tailored data.

Systems biology aims at analyzing assorted biological data (e.g., genomics, proteomics, metabolomics, fluxomics), and it has consistently assisted in understanding the complex underlying mechanisms in health and disease (Mardinoglu and Nielsen, 2012; Agren et al., 2014; Benfeitas et al., 2014; Ghaffari et al., 2015b; Mardinoglu et al., 2017). Using systems biology approaches, others successfully clarified the role of small molecular decision circuits (e.g., Faratian et al., 2009; Gaglio et al., 2011; Tyson et al., 2011) and found commonalities across different cancers through pan-cancer analyses (e.g., Weinstein et al., 2013; Zack et al., 2013; Leiserson et al., 2014; Aran et al., 2015; Peng et al., 2015). However, few studies have addressed ROS metabolism using systems approaches (Zhang et al., 2010; Zhou et al., 2011; Zhan et al., 2012). For instance, modeling of the NFE2L2 pathway suggests that the high NFE2L2 expression that typically occurs in cancer cells promotes chemoresistance (Zhan et al., 2012) and suggests apparently opposite roles in antioxidant and ROS-mediated cancer signaling (Zhang et al., 2010). However, certain antioxidants, such as peroxiredoxins, display both scavenging and redox sensor and signaling transducer properties (**Box 1**). Mathematical modeling of H_2O_2 metabolism indicates that cancer-related transcription factors are unlikely to be activated by direct reaction with H_2O_2, and it points toward protein thiols as the likely signaling sensors and transducers. Others have observed that the glutathione and NADPH synthesis pathways are simultaneously up- or down-regulated in breast, ovary, colon and lung cancers, establishing important interactions with *de novo* nucleotide synthesis (Mehrmohamadi et al., 2014), which suggests that cancers utilize redox homeostasis and biosynthesis pathways in parallel. ROS and redox responses of cancer cells have yet to be more extensively examined.

With the advent of big data, recent approaches aimed at understanding cell metabolism now incorporate large reaction networks derived from omics technologies (genomics, transcriptomics, proteomics, and others). By encompassing whole-cell reaction networks, GEMs have helped identify important redox alterations in metabolic diseases and physiological processes. For instance, disturbed H_2O_2 metabolism is observed (Mardinoglu et al., 2017) in non-alcoholic fatty liver disease, specifically due to deficient GSH biosynthesis (by GCLC/GCLM, GSR) and NADPH production (NNT). A lower abundance of plasma glycine, a substrate for *de novo* GSH biosynthesis, is also found in subjects with high hepatic steatosis. Observations in mice (Mardinoglu et al., 2015b) indicate that commensal gut microbes decrease glycine availability in the gastro-intestinal tract of the host, which results in decreased *de novo* GSH synthesis and promotes *NNT* and *GSR* expression, possibly to compensate for the decreased GSH pool. In the context of cancer, publicly deposited genomic data permit the stratification of cancer patients based on network-specific

mutations (Hofree et al., 2013), and these data have been used to find biomarkers and potential cancer therapeutic targets (Jerby and Ruppin, 2012; Agren et al., 2014). Others have combined experimental and interactome data with stochastic modeling to find that ROS and DNA damage are necessary and sufficient for senescent growth arrest (Passos et al., 2010). Chronic, non-toxic ROS supplementation reverses drug resistance in carcinoma cells (Maiti, 2010), which, through pathway analysis, identified several genes (e.g., *TP53*, Rac/Cdc42 guanine nucleotide exchange factor 6 *ARHGEF6*, and a DNA-activated protein kinase *PRKDC*) that mediate ROS-related apoptosis. Reconstruction of a generic human whole-cell GEMs encompassing >9,000 reactions and >3,000 metabolites and genes (Mardinoglu et al., 2014a) enabled context-specific integration and interpretation of cancer omics data. Comparisons between hepatocellular carcinoma and healthy liver samples using these models indicate that tumors display an increased abundance of NADPH-producing enzymes (e.g., ME1, G6PD, TALDO1, and TKT) and that H_2O_2 may be used as reporter metabolite in some of the patients (Bjornson et al., 2015). Personalized medicine approaches would greatly benefit from clarifying redox cancer responses. GEMs were previously used to identify novel anticancer drugs by reconstructing patient- and tissue-specific GEMs (Agren et al., 2014) and to identify anti-growth factors in human cancer cell lines (Ghaffari et al., 2015a). Cell-specific GEMs have also been used to identify malonyl-CoA decarboxylase, an important enzyme in fatty acid metabolism, as a selective and effective cancer therapeutic target (Yizhak et al., 2014a). Its inhibition drains reducing equivalents, decreases GSH/GSSG ratios, and promotes oxidative stress, which may help in chemotherapeutic approaches. Patient-specific breast and lung cancer GEMs were also able to predict patient survivability, and they predicted that knockout of *GSR* significantly affects cancer growth.

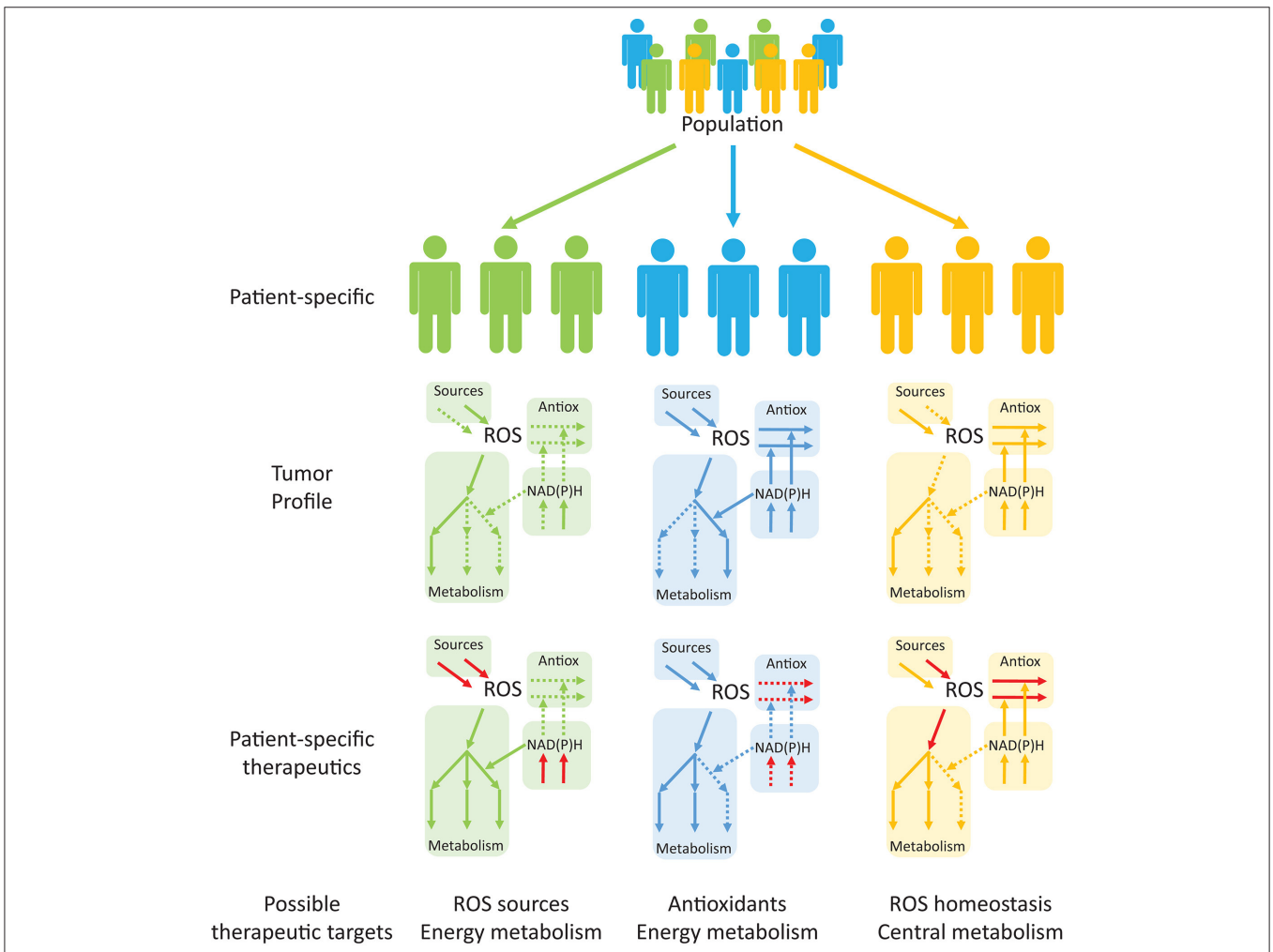

FIGURE 6 | Personalized systems medicine approaches are emerging as useful tools in devising patient-specific, rather than population-based, therapeutic targets in cancer. Tumor profiling of patients may help in identifying up- and down-regulated pathways (continuous and dashed arrows, respectively) that are suitable for therapeutic targeting. Drug targeting of specific processes (red arrows), either to promote or inhibit the processes, will permit alterations in the consequences of redox processes in cancer and other diseases.

Interestingly, despite an enzyme- or process-specific focus in redox cancer metabolism in recent years, system-wide studies of ROS and redox metabolism and their interactions with central metabolism in multiple cancers are lacking. Antioxidant profiling of tumor cells and their surrounding cells may be used in conjunction with patient-specific GEMs to identify the best therapeutic targets in this disease (**Figure 6**). Studies using reaction network information with tissue- and patient-specific models will be useful in (1) Redox profiling of patient-specific cancer tissues; (2) Understanding mechanistic properties of redox responses; (3) Devising effective, selective and patient-specific therapeutic strategies to regulate redox responses; (4) Establishing redox-based therapies to synergize with existent drugs (Kasiappan and Safe, 2016) and identifying and averting drug-resistance mechanisms; and (5) Shortening the gap between pre-clinical and clinical trials, potentially overcoming issues faced by previous trials, such as those that assessed the impact of dietary antioxidants on cancerogenesis (Omenn et al., 1996; Goodman et al., 2011; Klein et al., 2011; Sayin et al., 2014). The adopted strategies (**Figure 6**) will benefit from patient-specific tumor profiling to identify single or multiple targetable processes within the same pathway or to identify processes that serve as metabolic central hubs. The combination of these approaches with drugs that target other metabolic processes may promote desirable synergisms. Redox systems medicine is thus an interesting emerging field with potentially important implications for disease treatment.

CONCLUSION

Redox, energetic and central metabolism are closely intertwined, and the view that ROS are simple secondary products of cell metabolism is long gone. Together with their redox partners, ROS and antioxidant defenses are now regarded as crucial processes in tumorigenic initiation, progression and aggressiveness. However, the redox cancer responses are highly heterogeneous, manifesting not only between different cancer types but also between patients who suffer from the same cancer, and they are altered throughout cancer progression. The influence of ROS on different biochemical levels makes it necessary to seek an integrative analysis of these systems at the genomic, proteomic, and metabolomic levels. Approaches that are able to encompass these levels and integrate the crosstalk between antioxidant, redox, energetic, and central metabolism are able to capture and understand these complex responses. Systems biology approaches may be used to analyze omics data and understand the roles of each redox system in cancer. These approaches may be tissue- and patient-tailored, which enables the identification of the best therapeutic targets, while taking in account patient-specific oncogenic backgrounds. This is the aim of personalized systems medicine (Mardinoglu and Nielsen, 2015; Schork, 2015), an emerging field that presents a high potential to overcome some of the problems in therapeutic treatments, including the low (<25%) drug efficacies caused by population—rather than patient-wise data (Schork, 2015). Together with existing drugs, novel or existing redox-targeting drugs may be identified to produce synergistic responses for the treatment or prevention of cancer. Personalized medicine may thus enable an understanding of the role of redox systems in cancer and other diseases and may assist in drug discovery.

AUTHOR CONTRIBUTIONS

RB has written the manuscript. All authors actively contributed in writing and editing of the manuscript.

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Metabolism and Transcription in Cancer: Merging Two Classic Tales

Natalia Martín-Martín[1,2], *Arkaitz Carracedo*[1,2,3,4] *and Verónica Torrano*[1,2*]

[1] CIC bioGUNE, Bizkaia Technology Park, Derio, Spain, [2] Centro de Investigación Biomédica en Red Cáncer, Madrid, Spain, [3] IKERBASQUE, Basque Foundation for Science, Bilbao, Spain, [4] Biochemistry and Molecular Biology Department, University of the Basque Country (UPV/EHU), Bilbao, Spain

Cellular plasticity, or the ability of a cancer cell to adapt to changes in the microenvironment, is a major determinant of cell survival and functionality that require the coordination of transcriptional programs with signaling and metabolic pathways. In this scenario, these pathways sense and integrate nutrient signals for the induction of coordinated gene expression programs in cancer. This minireview focuses on recent advances that shed light on the bidirectional relationship between metabolism and gene transcription, and their biological outcomes in cancer. Specifically, we will discuss how metabolic changes occurring in cancer cells impact on gene expression, both at the level of the epigenetic landscape and transcription factor regulation.

Keywords: cancer metabolism, nutrient sensing networks, transcription factors, histone acetylation, DNA and histone methylation, gene expression regulation

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***Correspondence:**
Verónica Torrano
vtorrano@cicbiogune.es

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INTRODUCTION

The advances toward curative treatments for cancer are nowadays based on three pillars of research: (i) early detection, (ii) molecular stratification of high-risk patients and (iii) the selection of the most appropriate therapeutic strategy. New insights in the molecular understanding of cancer has led to a paradigmatic change in the way we combat the disease, introducing the concept of precision medicine: patient's stratification and personalized therapy.

In the recent years there has been a renaissance in the study of the cross-interaction between two important "usual suspects" in cancer: gene expression and metabolism (Hanahan and Weinberg, 2011). Both research areas have inherited potential to be applied to precision medicine. On the one hand, the study of transcriptional regulators can potentially lead to the development of stratification tools. On the other, the stratification can define which cancer patients will benefit from a given metabolic-based therapeutic approach (**Figure 1A**).

Along the process of transformation, the acquisition of pro-survival abilities is a crucial determinant that enables cancer cells to adapt to the ever-changing environment (Hanahan and Weinberg, 2011). This master adaptation is based, in part, on the connection between nutrient sensing and gene expression programs. As a consequence, cancer cells rewire their metabolism to activate the fittest metabolic rate for cancer homeostasis. This type of response requires a circuit in which cellular metabolism and gene transcription must be bidirectionally connected and tightly coordinated (**Figure 1B**).

One of the most important cellular regulatory mechanism that determine which genes are activated is the packing of DNA and histones in chromatin or epigenetic remodeling. Post-translational modifications of histones and DNA—mainly acetylation and methylation—alter the structure of chromatin, helping or preventing the recruitment of transcription factors complexes that will ultimately regulate gene expression. At the same time, changes in gene expression in

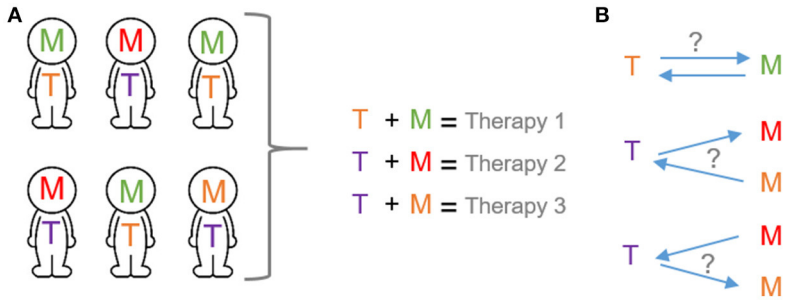

FIGURE 1 | Transcription and metabolic programs as potential tool for precision medicine. **(A)** The application of the precision medicine concept will depend on the selection of specific cancer therapies based on both transcriptional and metabolic programs of cancer patients. **(B)** Mechanistic basis of precision medicine. Bidirectional interplay between transcription and metabolic programs. T, transcription programs; M, metabolic programs. Each color exemplifies different programs.

response to environmental fluctuations are led by post-translational modifications or activation of transcription factors. Metabolism is the process of energy transduction that encompasses a network of chemical reactions tightly regulated by environmental changes. The idea that epigenetics and gene transcription can be influenced by products of metabolic pathways was proposed many years ago (Shi and Shi, 2004), but the biological relevance of this concept in tumorigenic processes has remained largely unknown.

Systematic profiling of cancer specimens has determined the existence of epigenetic alterations across the genome that potentially regulate gene expression and are associated with tumor progression (Baylin and Jones, 2011). This expanding field is coming together with cancer metabolism. During transformation, the entire metabolic network is rewired to efficiently convert nutrients to biosynthetic precursors to sustain cancer cell growth (Hanahan and Weinberg, 2011).

Metabolic and epigenetic enzymes are frequently components of the same tumorigenic pathway. Thus, metabolic rewiring occurring in cancer can impact on the regulation of chromatin structure and, therefore, cancer-related gene expression. Conversely, nutrient availability, or extracellular signals within the tumor microenvironment can fine-tune the expression of metabolic genes through epigenetic modifications and transcriptional regulation (**Figure 2**).

IMPACT OF METABOLISM AND ITS PRODUCTS IN GENE EXPRESSION PROGRAMS

Most chromatin-modifying enzymes use co-factors and substrates that are critical metabolites of the intermediary metabolism. The availability of these metabolites can influence the capacity of the cell to write or erase chromatin marks, highlighting the intimate link between the metabolic state, epigenetic regulation and gene expression.

DNA and Histone Methylation

In human DNA, cytosines are typically methylated at CpG islands located in promoter regions and associated with transcriptional regulation. Cancers frequently display global DNA hypomethylation but hypermethylation of CpG islands in genomic regions where tumor suppressor genes are located (Hansen et al., 2011). These histone methyl marks can either activate or repress gene expression (Kinnaird et al., 2016).

Methylation is linked to the intermediary metabolism through S-adenosyl methionine (SAM), the primary source of methyl groups that is generated in the folate and methionine cycles (Maddocks et al., 2016; Mentch and Locasale, 2016). The activities of both histone methyltransferases (HMT) and DNA methyltransferases (DNMT) depend on the levels of intracellular SAM which varies based on serine and methionine availability. The deprivation of these essential amino acids induce reversible and rapid changes in histone and DNA methylation, which in turn change the transcriptional landscape of cancer cells (Mentch et al., 2015; Maddocks et al., 2016). In the light of these data, the methionine cycle and the sensing of SAM availability provide a direct link between intermediary metabolism and chromatin state in cells.

Interestingly, system biology approaches have revealed methionine cycle and one-carbon metabolism gene networks as major determinants of DNA methylation status in human cancer and cancer survival predictors (Mehrmohamadi et al., 2016). Indeed, dysregulation of histone methylation in specific chromatin regions is a major selective force for tumor progression and metastatic potential (McDonald et al., 2017). Of note, the epigenetic changes associated with distant metastasis are strongly dependent on the oxidative branch of the pentose phosphate pathway (oxPPP). This dependency confers selective advantages to the disseminated cells enabling their metastatic spread. In distal metastasis sites, oxPPP is coupled to epigenetic programs that promote tumorigenesis (McDonald et al., 2017).

The demethylation reaction is also susceptible to metabolic fluctuations. The enzymatic removal of methyl groups is regulated by histone and DNA demethylases whose activities are modulated by the tricarboxylic acid (TCA) cycle intermediates alpha-ketoglutarate (α-KG), fumarate and succinate. When presented in sufficient concentration, α-KG acts as a positive co-factor of the demethylase activity, while fumarate and succinate are competitive inhibitors of multiple histone demethylases (Xiao et al., 2012). The activity of these enzymes can be dramatically

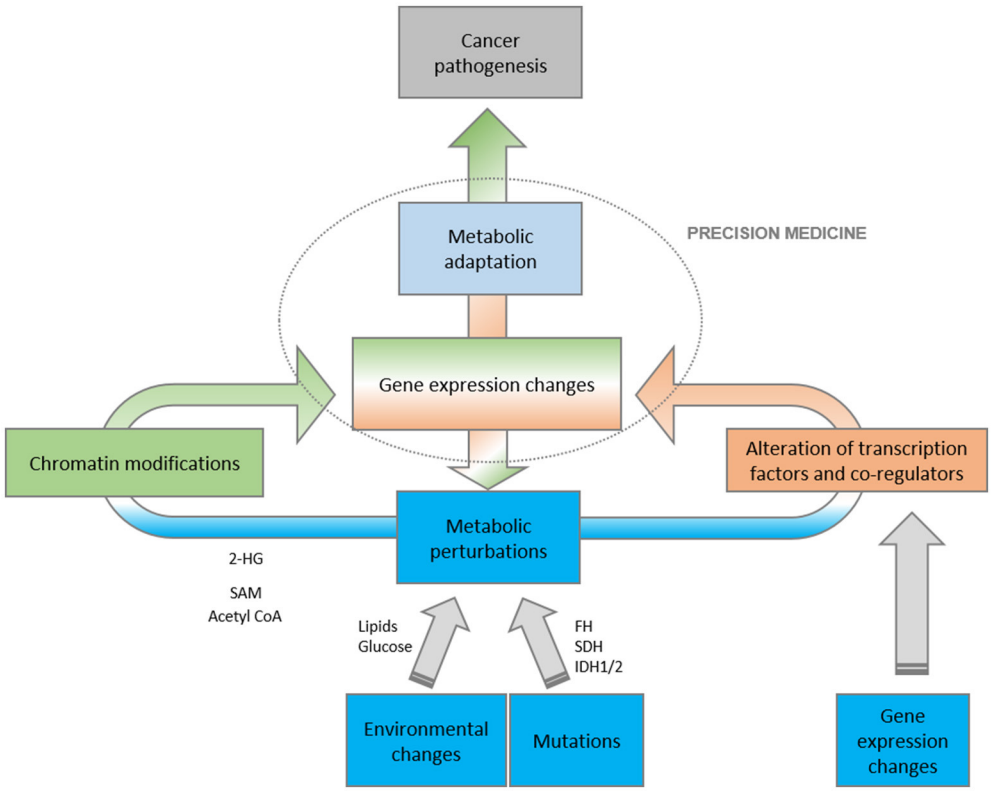

FIGURE 2 | Schematic representation of the complex relationship between metabolism and gene expression. Metabolic perturbations, as a result of environmental, mutational and metabolic insults, directly impact on gene expression programs, both at the level of epigenetic changes and transcriptional activities. The final outcome is that the conjunction of metabolism and transcription have a profound impact on oncogenesis.

altered by mutations in key metabolic enzymes. Inactivating mutations affecting the mitochondrial succinate dehydrogenase (SDH) complex subunits and fumarate hydratase (FH) are driver mutations in a subset of cancers (Tomlinson et al., 2002; Janeway et al., 2011; Pantaleo et al., 2011; Castro-Vega et al., 2014; Clark et al., 2014). These mutations lead to the accumulation of succinate and fumarate and the subsequent inhibition of α -KG-dependent dioxygenases (Xiao et al., 2012). The direct contribution of fumarate accumulation and epigenetics to tumorigenesis has been elegantly shown in the context of *FH* loss. In this scenario, fumarate accumulation elicits epigenetic changes in a regulatory region of the antimetastatic miRNA cluster *mir-200ba429*. In turn, the suppression of miR-200 leads to the expression of epithelial-to-mesenchymal-transition (EMT)-related transcription factors and the enhancement of migratory properties (Sciacovelli et al., 2016). Deficiency of SDH is associated with global DNA methylation changes (Killian et al., 2013) and the downregulation of neuroendocrine differentiation genes linked to a migratory phenotype (Letouze et al., 2013).

Upstream of SDH in the TCA cycle, isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate, producing α-KG and CO_2. IDH genes are the most frequently mutated metabolic genes in cancers driving global epigenetic changes (Figueroa et al., 2010; Ward et al., 2010; Cairns et al., 2012). Mutations in IDH1/2 have oncogenic properties and impede the synthesis of α-KG but favor the formation of the oncometabolite 2-hydroxyglutarate (2-HG) (Dang et al., 2009; Ye et al., 2013). In turn, 2-HG accumulation inhibits DNA demethylation (Losman et al., 2013) and primes cancer cells for transformation (Figueroa et al., 2010; Lu et al., 2012; Turcan et al., 2012). However, the production of 2-HG is not restricted to an IDH mutated background. For example, in hypoxia wild-type IDH2 produces 2-HG as a by-product (Wise et al., 2011). In ER-negative breast cancer patients, the accumulation of 2-HG define a subgroup of wild-type IDH2 patients with specific hypermethylation phenotype and poor clinical outcome (Terunuma et al., 2014). This work suggests that the metabolic-epigenetic axis could be reflected in tumor subtypes of clinical relevance.

Beyond cancer biology, but conceptually connected, 2-HG has been proposed to act as an immunometabolite that links the environmental context to immune fate and function through a metabolic–epigenetic axis (Tyrakis et al., 2016; Xu et al., 2017). Given the important role of the immune system in the maintenance of chronic inflammation during tumorigenic processes (Numasaki et al., 2003; Grivennikov et al., 2012), these results may have implications for tumor immunology.

In summary, the accumulation of succinate, fumarate, and 2-HG contribute to cancer progression and position the Krebs cycle as mitochondrial custodian of the methylome (**Figure 2**).

Histone Acetylation

Global levels of nuclear histone acetylation are sensitive to overall acetyl CoA levels. Acetyl CoA is a key intermediate of central metabolism, which not only fuels ATP production via the TCA cycle, but also functions as an essential building block for the synthesis of fatty acids and sterols, and importantly histone acetylation. Acetyl CoA is generated from catabolic pathways of intermediary metabolism and at the same time used by anabolic processes such as lipid synthesis. In mammalian cells, there are three major enzymes that generate acetyl CoA: acetate-dependent acetyl-CoA synthetase 2 (ACSS2), citrate-dependent ATP-citrate lyase (ACLY) and mitochondrial pyruvate dehydrogenase complex (PDC). The relative importance of ACSS2, ACLY and PDC for nuclear histone acetylation differs on the basis of the developmental state, disease, tissue type and even subcellular location.

ACLY is the primary enzyme responsible for the synthesis of acetyl CoA from glucose-derived citrate and connects oncogenic signals to histone acetylation (Wellen et al., 2009; Lee et al., 2014). In the absence of ACLY, under nutrient deprivation or stress conditions, cells upregulate ACSS2, enabling cancer cells to utilize acetate to sustain tumor growth (Comerford et al., 2014; Mashimo et al., 2014; Schug et al., 2015) by providing acetyl CoA for fatty acid and phospholipid synthesis and histone acetylation (Zhao et al., 2016). In addition, under hypoxic conditions, acetate mediates epigenetic changes that specifically activate a lipogenic program and promote cancer cell survival (Gao et al., 2016). Importantly, ACSS2 has been recently identified as a chromatin-bound factor that regulates and coordinates gene expression programs related to long-term spatial memory (Mews et al., 2017). This is the first evidence of the direct and causal contribution of ACSS2-derived acetyl CoA to epigenetic modulation and gene expression.

Lipid-derived carbons are also a bona fide physiological source of acetyl CoA for histone acetylation. The acetyl CoA produced via the activation of fatty acid oxidation (FAO) is selectively used by histone acetyl transferases located at gene locus where key lymphatic and lipid-specific genes reside (McDonnell et al., 2016; Wong et al., 2017). These studies expand the landscape of nutrient sensing and uncover how lipids and metabolism are integrated by epigenetic events that control gene expression. In a cancer scenario, the uptake of fatty acids—mediated by CD36—and their oxidation sustain cancer-initiating cells and promote metastasis. Interestingly, these metastasis-initiating cells with high expression of CD36 are defined by a lipid metabolism transcriptional signature (Pascual et al., 2017). Although no link with epigenetic changes have been associated with this phenotype, we could predict that lipid uptake, and presumably its oxidation could play and important role in cell survival and cancer progression by regulating the epigenetic and transcriptional landscapes.

Due to its biochemical properties, the biosynthesis of acetyl CoA is thought to occur in the subcellular compartment where it is required. Therefore, the localized production of acetyl CoA by spatial regulation of its enzymatic producers would confer a high degree of specificity to metabolic regulation of histone acetylation and gene expression.

In the mitochondria, acetyl CoA is the main product of FAO, branch chain amino acid catabolism and pyruvate oxidation through the activity of PDC. Although PDC has classically been localized to the mitochondria, under metabolic insults, functional PDC translocate to the nucleus. There, it generates a nuclear pool of acetyl CoA that increases the acetylation of core histones important for S phase entry (Sutendra et al., 2014). In line, spatial regulation of ACSS2 confers specificity to the metabolic regulation of histone acetylation and together with ACLY were found in the nucleus (Takahashi et al., 2006; Wellen et al., 2009). Importantly, the "on site" generation of ACSS2-derived acetyl CoA at specific chromatin domains favors histone acetylation of key genes involved in long-term spatial memory, autophagy, cell survival and tumorigenesis (Bulusu et al., 2017; Li et al., 2017a; Mews et al., 2017).

Interestingly, the modulation of the mitochondrial protein VDAC1 induced a coordinated cascade of changes in mitochondrial metabolites that elicited a global metabolic re-programming in glioblastoma cells. This metabolic rewiring led to the activation of neural cell differentiation transcriptional programs and reversal oncogenic properties of glioblastoma cells (Arif et al., 2017).

In summary, chromatin-associated enzymes sense intermediary metabolism products and process this information into dynamic chromatin modifications that will ultimately regulate adaptive transcriptional programs associated with oncogenic processes.

TRANSCRIPTIONAL REGULATION OF METABOLIC PROGRAMS

The metabolic switch in cancer encloses a plethora of discrete enzymatic activities that must be coordinately altered in order to ensure the adaptation of cancer cells to environmental alterations (Loo et al., 2015). In the recent years, numerous reports have provided evidences of the cues regulating one or few enzymes within a metabolic pathway in cancer. However, the means of coordinated regulation of complex metabolic networks is starting to be elucidated (Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

Nutrients perturbations can be sensed directly by master transcriptional regulators of metabolism that will ultimately elicit the coordinated expression of genes required for metabolic adaptation in cancer cells (**Figure 2**). These programs allow the rapid adaptation to new biological states or external insults, and their contribution to cancer pathogenesis and progression has begun to emerge (Mouchiroud et al., 2014). More than fifty years ago an association between lipid metabolism and tumor progression was reported (Weinhouse et al., 1951) and since that time, the involvement of lipid metabolism in tumorigenesis has been thoroughly investigated.

Peroxisome-proliferator-activated receptors (PPARs), PPAR-α, PPAR-δ (also known as PPAR-β) and PPAR-γ, are members

of the nuclear receptor superfamily of transcription factors that control lipid sensing and the transcriptional regulation of metabolic pathways (Michalik et al., 2006). PPARs regulate gene expression programs that impact on proliferation, differentiation and survival, thus controlling carcinogenesis in various tissues including liver, breast, lung, colon and bone marrow. The role of these nuclear factors in transformation has been controversial during the past years, being described as either tumor suppressor or oncogenes (Carracedo et al., 2012; Ito et al., 2012; Peters et al., 2015; Lakshmi et al., 2017; Martin-Martin et al., 2017; Sun et al., 2017). The activity of PPARs is modulated upon ligand binding and by a number of coactivator and corepressor proteins, the presence of which can stimulate or inhibit the transcriptional function of the receptor (Feige and Auwerx, 2007; Martin-Martin et al., 2017). One of the most studied co-regulators of PPARs function is PPAR gamma co-activator 1 alpha (PGC1α), a master transcriptional co-activator with broad functions in energy metabolism. Together, PPARs and PGC1α control mitochondrial function and FAO (Sugden et al., 2010) and have been implicated in the maintenance of hematopoietic stem cell pool, cancer survival and progression (Carracedo et al., 2012; Ito et al., 2012; Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

The classical nuclear receptors are known as the receptors for steroids such as estrogen, androgen, glucocorticoid, and progesterone, which are derivatives of cholesterol. Among these classical nuclear factors, the sterol regulatory element binding-proteins (SREBPs) are the master transcription factors that are highly sensitive to the intracellular levels of cholesterol. The cholesterol composition of cellular membranes is an essential metabolic requirement for cell division (Bengoechea-Alonso and Ericsson, 2016). Different cancer cell types adapt their metabolism to maintain high intracellular cholesterol levels through increased cholesterol uptake and the activation of lipogenic transcriptional programs dependent on SREBP-1 (Guo et al., 2011; Huang et al., 2012; Li et al., 2017b). These pathways converge into the accelerated endogenous production of cholesterol. It has been recently described the regulation of ACSS2 by SREBP in mammary epithelial cells, having this regulation an effect on fatty acid synthesis (Xu et al., 2018). Given the important role of ACSS2 as a central node between metabolism and epigenetic regulation in cancer, it is tempting to speculate that the cholesterol levels in cancer cells may have an impact on gene regulation through the modulation of ACSS2 enzymatic activity.

The transcriptional agonist properties of cholesterol are not limited to SREBPs. Cholesterol has been recently identified as a physiological and functional endogenous agonist of the estrogen-related receptor alpha (ERRα). Upon cholesterol binding, ERRα recruits PGC1α coactivators to DNA promoters and together serve as a critical metabolic sensors that regulate gene expression programs associated to osteogenesis, myogenesis and macrophage activation (Wei et al., 2016). This is the first evidence for cholesterol and the cholesterol biosynthetic pathway in the regulation of ERRα activity and biology.

Taken together, all these data position cholesterol as a master metabolite that control gene transcription programs via its interaction with nuclear factors.

ERRα and its transcriptional programs are implicated in metabolism and cancer progression. Increased ERRα activity is observed in melanoma, breast and ovarian cancer, colorectal carcinoma and osteosarcoma (Stein and McDonnell, 2006; Vazquez et al., 2013; Chen et al., 2014; Thewes et al., 2015). ERRs are nuclear receptors that exhibit ligand-dependent regulation, and their activity relies on the status of transcriptional co-activators and co-repressors (Feige and Auwerx, 2007). One such co-activators, PGC1α has been extensively studied in physiological conditions (Handschin, 2009). PGC1α controls transcriptional programs that increase the energetic yield (Scarpulla, 2011) and counteract oxidative stress (St-Pierre et al., 2006; Haq et al., 2013; Vazquez et al., 2013), which enables elevated oxidative mitochondrial activity (OXPHOS) coping with the accumulation of reactive oxidant species (ROS). PGC1α exerts paradoxical activities in different tumor types and biological conditions and recent studies highlight the importance of it in cancer metabolism (Vazquez et al., 2013; LeBleu et al., 2014; Sancho et al., 2015; Luo et al., 2016) and specifically through the regulation of ERRs (Haq et al., 2013; Vazquez et al., 2013; Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

The classical view of cancer metabolic wiring (Warburg effect) would predict that the PGC1α-ERRα axis and OXPHOS triggered are inherently tumor suppressive. However, recent studies uncover that factors such as mutational background, tissue or cell of origin and disease stage impose a pressure toward the best-adapted metabolic wiring during cancer progression. In melanoma and breast cancer, cells turn on PGC1α and their OXPHOS program, which impacts on cancer cell survival, proliferation and contribution to therapy resistance (Haq et al., 2013; Vazquez et al., 2013; LeBleu et al., 2014). Interestingly, during the process of metastasis, melanoma cells need to suppress PGC1α expression in order to regulate an adhesion and invasion transcriptional program (Luo et al., 2016). In line, OXPHOS PGC1α-induced metabolism represents a disadvantageous metabolic state in prostate cancer. Moreover, the decrease of PGC1α-ERRα transcriptional activity provides a selective advantage to metastasize and correlates with an increased disease recurrence (Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

These studies elegantly illustrate how the PGC1α-ERRα transcriptional axis can exert opposing activities in cancer progression, highlighting the metabolic diversity leading to metabolic adaptations during cancer progression in different cancer types.

CONCLUDING REMARKS

Metabolic rewiring and gene deregulation are both hallmarks of cancer (Hanahan and Weinberg, 2011) and are addictive for tumor cells (Bradner et al., 2017; Vander Heiden and DeBerardinis, 2017). Thus, the crosstalk between gene expression and metabolism are fundamental aspects of cellular adaptation to nutritional changes during tumorigenesis. An attractive approach to understand cancer and identify therapeutic targets is to discover the key components on which deregulated transcriptional and metabolic programs depend in cancer cells. We have outlined recent advances

that described how coordinated gene expression programs are tightly and dynamically regulated by the metabolome, either at the level of chromatin modifications and transcription factor activities. In this scenario, metabolic alterations during cellular transformation drive aberrant gene expression which in turn will be key contributors to tumor development and progression. However, much remains to be discovered, and the study of the bidirectional contribution of metabolism to gene expression regulation will bring a more integrated understanding of cellular adaptations during cancer progression and, possibly new therapeutic opportunities.

AUTHOR CONTRIBUTIONS

VT wrote the manuscript with essential contribution of AC and NM-M.

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MINI REVIEW
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Aberrations of DNA Repair Pathways in Prostate Cancer: Future Implications for Clinical Practice?

Orazio Caffo, Antonello Veccia, Stefania Kinspergher, Mimma Rizzo and Francesca Maines*

Department of Medical Oncology, Santa Chiara Hospital, Trento, Italy

Patients who are carriers of inherited mutations in essential component of DNA repair pathways have a significantly higher lifetime risk for developing cancer compared to the population of reference. Recent advances in DNA next-generation sequencing technology have allowed screening for carriers of those mutations, allowing development of promising risk-reduction strategies and providing the rationale to personalize the therapeutic approach for these patients. New intriguing scenarios are opening nowadays for the management of prostate cancer in patients with germline or somatic mutations in components of DNA repair pathways (e.g., *BRCA1* and *BRCA2* genes), such as specific screening policies and new therapeutic strategies involving PARP inhibitors or platinum-based chemotherapy.

Keywords: prostate cancer, PARP inhibitors, BRCA1, BRCA2, olaparib

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****Correspondence:***
Orazio Caffo
orazio.caffo@apss.tn.it

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INTRODUCTION

BRCA1 and *BRCA2* are tumor suppressor genes with essential functions in the maintenance of genome stability (Yoshida and Miki, 2004). Both genes are characterized by an autosomal dominant inheritance pattern with incomplete penetrance, and individuals with heterozygous germline mutations in BRCA1/2 genes are at risk of losing the functional allele as a consequence of a second damage induced by alkylating agents, ionizing radiation, reactive oxygen species or chemical mutagens (Evers et al., 2010). The functional loss of *BRCA1 or 2* leads to a defect in double-strand breaks repair through the homologous recombination process, which, in turn, drastically affects the ability of the cell to preserve genome fidelity and stability (Liu and West, 2002).

It is well established that women who are carriers of inherited harmful *BRCA 1* or *2* gene mutations have an increased risk to develop breast and/or ovarian cancer in lifetime compared to their wild-type counterparts (Easton et al., 2007). Importantly, these cancer susceptibility genes have been associated to an increased risk of developing several other types of tumor, such as fallopian tube and peritoneal cancer in women (Brose et al., 2002; Finch et al., 2006), breast and prostate cancer (PC) in men (Levy-Lahad and Friedman, 2007; Tai et al., 2007; Mersch et al., 2015) and pancreatic cancer in both sexes (Ferrone et al., 2009; Mersch et al., 2015) although not fully overlapping results were observed (van Asperen et al., 2005; Moran et al., 2012).

BRCA AND PROSTATE CANCER

The incidence of germline BRCA mutations in newly diagnosed PC, unselected for family predisposition, ranges approximately from 1.2 to 2% of the cases (Leongamornlert et al., 2012).

BRCA carriers have an increased risk to develop PC (at 1.8- to 4.5-fold for BRCA1 carriers and at 2.5- to 8.6-fold for BRCA2 carriers in patients aged <65 years), in particular at early onset (Kote-Jarai et al., 2011; Leongamornlert et al., 2012).

Management of high-risk men with germline mutations in DNA-repair genes is uncertain and controversial, without consensus on the screening for PC. In this population, the harms of overdiagnosis and overtreatment are mitigated by the increased incidence and risk of PC-specific mortality. The National Comprehensive Cancer Network guidelines recommend that men with BRCA mutations should perform breast self-examination starting at 35 years of age and annually thereafter. At 45 years of age BRCA2 carriers should begin PSA screening and BRCA1 carriers should be advised to consider it. Prostatic biopsy is recommended at PSA > 3.0 ng/mL in this population [15].

The IMPACT study (**I**dentification of **M**en with a genetic predisposition to **P**rost**A**te **C**ancer: **T**argeted screening in BRCA1/2 mutation carriers and controls) evaluated a tailored PC screening in 1522 men with BRCA1/2 germline mutation, proposing annual PSA tests and a prostate biopsy if PSA >3 ng/mL (Bancroft et al., 2014). Despite the lack of statistically significant difference in PC detection rate between carriers and controls, the authors observed a higher incidence of PC in BRCA2 carriers compared to BRCA1 and controls (3.3 vs. 2.6% and <2%, respectively); in addition more than 2/3 of the PCs detected in the BRCA2 carriers and 61% in BRCA1 carriers were classified as intermediate or high risk. In light of these results, they suggested that mainly in BRCA2 carriers PSA testing should be proposed earlier, repeated at shorter screening intervals, and with lower PSA thresholds compared to the general population, in order to detect the tumorigenic transformation earlier. It is clear that according to the actual limitations of PSA-based screening, final results of the study are waiting to define the optimal screening program in this subset of patient, although the preliminary results seem to support PSA routine testing in BRCA carriers (Mitra et al., 2011).

An ongoing clinical trial in Toronto, Canada (NCT01990521) is evaluating the role of prostate MRI in male BRCA carriers, regardless of the PSA values.

BRCA2 germline mutations in men with PC have been associated with an aggressive tumor phenotype, a more advanced tumor stage at the diagnosis and poor survival outcomes at any disease stage, including the localized/locally advanced disease (Tryggvadottir et al., 2007; Castro et al., 2013). In particular, it has been recently observed that BRCA mutation carriers with localized PC have worse outcomes than those who are wild type regardless of the local treatment they have previously undergone (radical prostatectomy or radiation therapy; Castro et al., 2015). The 5-year metastasis-free survival was significantly higher in wild-type patients compared to mutation carriers (93 vs. 77%; $p = 0.009$). BRCA carriers had higher rate of lymph-nodes involvement, higher Gleason score, developed distant metastasis earlier and had a shorter survival. Overall, the independent prognostic value of this mutation at the multivariate analysis strongly suggests the need of a timely management in BRCA carriers (Castro et al., 2013, 2015). Furthermore, these patients developed more frequently castration-resistant PC (CRPC) upon occurrence of metastases (Castro et al., 2013).

Overall, several studies suggested that BRCA mutation is an independent negative prognostic factor for both overall survival and PC-specific survival (Modena et al., 2016).

Taken together, all these findings suggest that active surveillance may not be a valid treatment option for BRCA mutation carriers, even in the low-risk PC population, according to more aggressive behavior and poor disease outcomes observed in such subjects. For all these reasons screening for BRCA1/2 might be useful in early diagnosis and potentially have a beneficial impact on the management of these patients.

Aberrations in genes involved in DNA integrity seem to increase in the late-stages of PC disease with 20-30% men with metastatic CRPC (mCRPC) carrying genomic defects in DNA-repair pathways (Mateo et al., 2017). At the moment it is not clear if the increased incidence of defects in DNA-repair in mCRPC is related to progression to a more aggressive disease phenotype or rather it is the result of a secondary pressure due to specific treatments.

IMPLICATIONS FOR THE TREATMENT

In the last decades the therapeutic landscape of mCRPC patients has dramatically changed due to availability of several agents able to significantly improve survival: chemotherapeutic agents, docetaxel (Tannock et al., 2004) and cabazitaxel (de Bono et al., 2010), new generation hormone agents, abiraterone (de Bono et al., 2011; Ryan et al., 2014), and enzalutamide (Scher et al., 2012; Beer et al., 2014), and one radiopharmacetical agent, radium 223 (Parker et al., 2013).

In addition, there is a growing interest for strategies based on the immunotherapy: after the studies on vaccines leading to the FDA approval of sipuleucel-T (Kantoff et al., 2010a) or to the development of PROSTVAC-VF (Kantoff et al., 2010b), immune-checkpoints inhibitors are currently being tested in mCRPC, such as pembrolizumab (NCT02787005) and atezolizumab in combination with enzalutamide (NCT03016312).

The use of targeted therapies such as poly-(ADP-ribose) polymerase (PARP) inhibitors in BRCA-associated breast and ovarian cancers (Audeh et al., 2010; Tutt et al., 2010; Ledermann et al., 2012) suggests a potential role of these drugs also in BRCA carriers affected by other solid tumors, including PC. PARP polymerase is a nuclear DNA-binding enzyme involved in the single-strand break DNA repair, through the base excision and repair (BER) pathway (Morales et al., 2014). Impairment of BER activity through PARP inhibition determines the so called *synthetic lethality* interaction in homologous recombination deficient BRCA-mutant cancer cells ("BRCA-ness"), an overwhelming genome instability condition which drives cancer cells to die (Farmer et al., 2005). BRCAness tumors seem to be highly sensitive to PARP inhibitors, independently of the site of origin of the tumor (Underhill et al., 2011).

Several PARP inhibitors (olaparib, rucaparib, niraparib, velaparib, and talazoparib) are currently being investigated in

several tumor types. In the case of BRCAness PCs, phase I clinical trials (Fong et al., 2009; Sandhu et al., 2013a), small mCRPC series (Sandhu et al., 2013b), and translational studies (Brenner et al., 2011) suggested a role for these agents also in PC patients. Orally administered Olaparib at 400 mg twice a day was tested in 298 patients with germline BRCA1/2 mutation and recurrent advanced cancers, including eight pre-treated PC patients (Kaufman et al., 2015). Among these PC patients, seven had a BRCA2 mutation and the remaining was a BRCA1 carrier. All these patients had previously received an average of two lines of treatment for the advanced disease. Although the very limited number of PC patients, the results were encouraging since 4 out of 8 patients showed tumor response while in 2 the disease remained stable. The median progression-free survival (PFS) was 7.2 months with two patients having a favorable response for longer than 1 year. It is noteworthy that only one out of four PC patients who had previously received platinum chemotherapy responded to the PARP inhibitor, suggesting a potential cross-resistance between the mechanisms of action of these drugs (Kaufman et al., 2015).

On the basis of these promising results, the study TOPARP-A (a larger phase II clinical trial) investigated the activity of Olaparib in 50 mCRPC patients who had shown progression disease after one or two treatments, including docetaxel (Mateo et al., 2015). In this study genomic defects in DNA-repair genes were prospectively evaluated with next-generation sequencing analyses on fresh tumor-biopsy performed before the treatment. The primary endpoint of the study was composite: radiological response according to RECIST 1.1 and/or PSA declines >50% and/or conversion in circulating tumor cells (CTC). PFS and OS were secondary endpoints of the study. A response to Olaparib was observed in 16 patients (33%), who received the drug for 6 months in 12 cases and for 12 months in four cases. The median OS was 10.1 months (5.1–15.6). Molecular analyses identified aberrations in DNA-repair genes [BRCA 1/2, ataxia-telangiectasia mutated (ATM), Fanconi's anemia genes, CHEK2, PALB2, FANCA, HDCA2, and others] in 16/49 patients. Of these 16 biomarker-positive patients, 14/16 (88%) showed a response to Olaparib, of particular relevance, 7/7 patients with BRCA2 mutation and 4/5 patients with ATM aberrations. The PSA response in those who had a clinical benefit from PARP inhibitors (13/16, 81%) suggested that PSA monitoring during the treatment could be useful to rapidly identify the responders. Radiologic PFS and OS were significantly longer in the biomarker-positive compared to the biomarker-negative group (median 9.8 vs. 2.7 months, $p < 0.0001$; median 13.8 vs. 7.5 months, $p = 0.05$, respectively; Mateo et al., 2015).

The tolerability profile of the drug was manageable and mainly related to hematological toxicities (anemia, thrombocytopenia), fatigue and gastrointestinal side effects. The striking results in BRCA1/2 and ATM gene-mutated mCRPC patients led the US Food and Drug Administration (FDA) to approve this breakthrough therapy with Olaparib for this population, although efficacy and safety results of the phase II trial need to be confirmed in larger trials. Noteworthy, it could be reductive to restrict treatment with Olaparib to patients with BRCA or ATM mutations only since this drug showed to be effective in additional 25% of patients who are very likely carriers of unknown defects in homologous recombination.

The Part B of TOPARP study (NCT01682772) (TOPARP-B) aims to validate the role of Olaparib in BRCA2 or ATM carriers and to provide additional efficacy data in presence of less common mutations in other genes involved in DNA repair such as FANC, CDK12, RAD51, PALB2, ATR, CHEK1, CHEK2, DSS1, MRE11, XRCC2/3, and ETS gene fusions (TMPRSS2-ERG) which have been previously linked to PARP inhibitors sensitivity (McCabe et al., 2006; Yang et al., 2011; Hussain et al., 2014).

Additional studies are waiting to confirm that the frequency of DNA-repair defects in mCRPC patients is higher than that observed in other disease settings or in untreated patients. Preliminary results suggest that somatic BRCA mutations are more often observed in late stages of prostate cancer disease; for this reason, in the next future, genomic re-assessment of the disease with a new fresh biopsy or using isolated circulating cells or circulating DNA will become desirable to personalize the therapeutic approach.

Mateo J et al. retrospectively reviewed the clinical outcome of mPC patients with and without germline DNA damage repair gene mutation (gDDRm); medical records were reviewed for 390 mPC patients with known gDDRm status. Data suggested that mPC patients with inherited mutations in DDR genes, including those with BRCA2 mutations, can achive similar benefit from standard of care therapies in terms of both response rate and PFS compared to patients without mutations [38].

Additional clinical trials are testing efficacy and safety of PARP inhibitors in combination with chemotherapy, radiotherapy or biological agents in several disease setting, including the localized disease. Phase II clinical trials are evaluating the combination of olaparib with abiraterone vs. placebo in mCRCP (NCT01972217), the combination of veliparib with abiraterone vs. abiraterone (NCT01576172) or the association of niraparib with enzalutamide (NCT02500901).

Another study evaluated the combination of veliparib (ABT-888) plus temozolamide in 26 mCRPC patients pre-treated with docetaxel: the authors demonstrated a very modest efficacy of the combination therapy with 12% of the patients achieving a PSA response >30% within 3 months (Hussain et al., 2014). Median PFS and OS were 9 weeks and 39.6 weeks, respectively. Hematological toxicities were observed; in particular, grade III/IV thrombocytopenia was noted in 15% of the patients. Despite the promising preclinical activity, this combination demonstrated disappointing results. The authors suggest that the administration of a low, sub-optimal dose of veliparib in this trial could explain the limited activity.

As observed in BRCA carriers patients affected by breast or ovarian cancer (Ahn et al., 1997; Yang et al., 2011), also carriers of mutations in DNA repair pathways could benefit from platinum-based chemotherapy and recent observations seem to support this hypothesis.

Cheng and colleagues reported some cases of very good response (complete or partial response) to platinum

chemotherapy in advanced prostate cancer (Cheng et al., 2016). Retrospective DNA sequencing of these patients demonstrated a biallelic inactivation of BRCA2.

Additionaly, the retrospective multicentre analysis from Pomerantz et al confirmed that mCRPC carriers of BRCA2 mutations have a higher likelihood of positive response to carboplatin-based chemotherapy than non-carriers (Pomerantz et al., 2017). These authors retrospectively assessed a cohort of 141 mCRPC patients treated with carboplatin and docetaxel and found that 75% of the 8 BRCA2 carriers showed a PSA decline >50% compared with 17% of the 133 non-carriers.

It is clear that the studies evaluating the efficacy of platinum-based therapies in selected subgroup of patients with defects in DNA-repair genes are necessary, but the detection of mutations in DNA-repair genes could represent a predictive biomarker able to drive the therapeutic strategy. Despite the number of agents efficacious in mCRPC patients, today no robust available biomarkers are able to predict the response to a specific class of agents. Growing retrospective data could suggest a reduced activity of new hormone agents compared to chemotherapeutic agents in presence of the splice variant of androgen receptor AR-V7 (Antonarakis et al., 2014, 2015; Scher et al., 2016). Unfortunately, to date, the expression of this biomarker has only a prognostic value, since only prospective randomized trials will be able to assess its predictive value.

CONCLUSIONS

Carcinogenesis is mediated by the accumulation of inherited or acquired genetic aberrations that promote the tumor growth advantage. DNA-repair defects can lead to an increase in genetic changes in cells resulting in an improved risk of developing cancer. The identification of the carriers of these genomic aberrations allows not only to identify people who have cancer susceptibility but also to define cancer subtypes with a different sensitivity to the treatments. It is likely that DNA sequencing will change the therapeutic approach to prostate cancer in the next years, improving molecular classification of this tumor and therefore the personalized therapeutic approach. Molecular characterization of prostate cancer seems to be promising to define also cancer prognosis.

In order to maximize the efficacy of cancer therapies avoiding unnecessary side effects, identification and prospective validation of predictive biomarkers are strongly advocated. In this context there is the need of carefully designed clinical trials which will be able to guide the tailored therapeutic approach and thus the clinical decision making process.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Oncology

ORIGINAL RESEARCH
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Synergistic Protective Activity of Tumor-Specific Epitopes Engineered in Bacterial Outer Membrane Vesicles

*Alberto Grandi[1], Michele Tomasi[2], Ilaria Zanella[2], Luisa Ganfini[2], Elena Caproni[2], Laura Fantappiè[2], Carmela Irene[2], Luca Frattini[2], Samine J. Isaac[2], Enrico König[2], Francesca Zerbini[2], Simona Tavarini[3], Chiara Sammicheli[3], Fabiola Giusti[3], Ilaria Ferlenghi[3], Matteo Parri[2] and Guido Grandi[2]**

[1] Toscana Life Sciences, Siena, Italy, [2] Centre for Integrative Biology (CIBIO), University of Trento, Trento, Italy, [3] GlaxoSmithKline Vaccines (GSK) Vaccines, Siena, Italy

Introduction: Bacterial outer membrane vesicles (OMVs) are naturally produced by all Gram-negative bacteria and, thanks to their plasticity and unique adjuvanticity, are emerging as an attractive vaccine platform. To test the applicability of OMVs in cancer immunotherapy, we decorated them with either one or two protective epitopes present in the B16F10EGFRvIII cell line and tested the protective activity of OMV immunization in C57BL/6 mice challenged with B16F10EGFRvIII.

Materials and methods: The 14 amino acid B cell epitope of human epidermal growth factor receptor variant III (EGFRvIII) and the mutation-derived CD4+ T cell neo-epitope of *kif18b* gene (B16-M30) were used to decorate OMVs either alone or in combination. C57BL/6 were immunized with the OMVs and then challenged with B16F10EGFRvIII cells. Immunogenicity and protective activity was followed by measuring anti-EGFRvIII antibodies, M30-specific T cells, tumor-infiltrating cell population, and tumor growth.

Results: Immunization with engineered EGFRvIII-OMVs induced a strong inhibition of tumor growth after B16F10EGFRvIII challenge. Furthermore, mice immunized with engineered OMVs carrying both EGFRvIII and M30 epitopes were completely protected from tumor challenge. Immunization was accompanied by induction of high anti-EGFRvIII antibody titers, M30-specific T cells, and infiltration of CD4+ and CD8+ T cells at the tumor site.

Conclusion: OMVs can be decorated with tumor antigens and can elicit antigen-specific, protective antitumor responses in immunocompetent mice. The synergistic protective activity of multiple epitopes simultaneously administered with OMVs makes the OMV platform particularly attractive for cancer immunotherapy.

Keywords: bacterial outer membrane vesicles, cancer immunotherapy, EGRRvIII, cancer neoepitopes, BALB/c-CT26 cancer mouse model, precision medicine

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****Correspondence:***
Guido Grandi
guido.grandi@unitn.it

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INTRODUCTION

All cancer therapies attempt to exploit the differences existing between tumor and normal cells. Since our immune system is built to target and destroy the "non-self," theoretically cancer vaccination is the safest, most natural, and effective therapeutic approach against cancer. Indeed, a large number of preclinical and clinical studies involving cancer vaccines have been described over the last two

decades. Unfortunately, in the clinical settings, the results so far have been disappointing. Klebanoff et al. (1) reported a cumulative analysis of several vaccine trials run from 2004 to 2009 and included 936 patients with different types of solid tumors. Using response rate as a measure of positive outcome, the conclusion of the study was that only 3.6% of the patients had an objective benefit from vaccination. The authors concluded that for cancer vaccines to become effective the strategies so far used for their formulation need be substantially revisited.

An ideal cancer vaccine should include three elements: (1) a cocktail of tumor-specific and/or tumor-associated antigens (TSA/TAAs), (2) one or more potent immune-stimulatory molecules (adjuvants), and (3) a delivery system which allows the co-delivery of cancer antigens and adjuvant(s) to antigen presenting cells (APCs). The absence of just one of these elements can make the vaccine incapable of counteracting the corrupted tumor microenvironment (containing regulatory T cells and aberrantly matured myeloid cells), and the highly mutable tumor targets (driving antigen loss and immune evasion).

Enthusiasm for therapeutic cancer vaccines has been recently rejuvenated by two major discoveries. First, it has been shown that the large number of mutations occurring in most tumors (2) creates "neo-epitopes," which can become the targets of both CD4+ and CD8+ T cells. Neo-epitope-specific T cells have been found among tumor-infiltrating lymphocytes (TILs), and when amplified *ex vivo* from tumor biopsies and introduced back into patients, TILs can exert antitumor activities (3). Moreover, the impressive therapeutic effect of checkpoint inhibitor antibodies observed in a fraction of patients has been shown to correlate with the number of tumor-associated mutations (4–6). Consequently, vaccines formulated with neo-epitopes have recently been created and shown to be highly effective in preventing tumor growth in different preclinical settings (7). Second, Kranz and coworkers (8) have demonstrated that when administered intravenously (i.v.) in melanoma patients, negatively charged liposomes carrying TSA encoding synthetic RNAs were efficiently taken up by splenic DCs, resulting in a potent elicitation of TAA-specific CD4+ and CD8+ T cells. Overall, these data support the hypothesis that therapeutic cancer vaccines can drive protective antitumor immune responses as long as specific TSAs/TAAs are formulated with the appropriate combination of adjuvant(s) and delivery system.

In our laboratories, we have become interested in bacterial outer membrane vesicles (OMVs) both from a scientific and translation viewpoint. More than 40 years ago, researchers made the observation that all Gram-negative bacteria release OMVs, closed spheroid particles, 20–300 nm in diameter, generated through the "budding out" of the outer membrane (9, 10). Consistent with their origin, the majority of OMV components are represented by lipopolysaccharide (LPS), glycerophospholipids, and outer membrane and periplasmic proteins (11, 12). OMVs have a multitude of functions, including inter and intra species cell-to-cell cross-talk, biofilm formation, genetic transformation, defense against host immune responses, and toxin and virulence factor delivery to host cells (11). From a translational standpoint, OMVs can be an attractive vaccine platform for three main reasons. First, they carry many microbe-associated molecular patterns, including LPS, lipoproteins, peptidoglycan, and flagellin, which by binding to pathogen recognition receptors play a key role in stimulating innate immunity and promoting adaptive immune responses (13–15). Such stimulatory molecules can work synergistically, thus potentiating the built-in adjuvanticity of OMVs (16). Second, OMVs can be easily decorated with foreign antigens/epitopes by manipulating the OMV-producing strain through different Synthetic Biology approaches. This feature was demonstrated for the first time by Kesty and Kuehn who showed that *Yersinia enterocolitica* outer membrane protein Ail assembled on OMV surface when expressed in *Escherichia coli*, and that the Green Fluorescence Protein fused to the "twin arginine transport" signal sequence was incorporated in the OMV lumen (17). Following this observation, an increasing number of heterologous proteins have been successfully delivered to OMVs using a variety of strategies (16, 18). Recently, we showed that different bacterial antigens could be delivered to the lumen of *E. coli* vesicles by fusing their coding sequences to a leader peptide for secretion (19). Moreover, we showed that heterologous lipoproteins could be incorporated into the OMV membrane and that such proteins could serve as chaperones to transport heterologous polypeptides to the OMV surface (20). Third, OMVs can be rapidly and easily purified from bacterial culture supernatant. The original OMV production methods, currently in use at industrial scale for *Neisseria meningitidis* group B vaccines, involve the treatment of bacterial biomass with mild detergents (21). More recently, detergent-free methods for OMV production have been proposed which make use of mutant strains featuring a hyper-vesiculating phenotype (19, 22–25). Once the supernatant is separated from the biomass of these mutant strains, the purification of the vesicles can be easily carried out using tangential flow filtration with production yield higher than 100 mg of vesicles (protein content) per liter of culture (26).

In this work, we addressed two main questions. First, we were interested to know whether OMVs decorated with a well-known, B cell cancer-specific epitope could induce epitope-specific immune responses and whether such responses could protect immunocompetent mice from the challenge with a syngeneic cancer cell line expressing the epitope on its surface. Second, we wanted to investigate whether the addition of a second cancer-specific epitope also expressed in the same cell line could result in a synergistic effect, thus potentiating the overall efficacy of the OMV cancer vaccine. As a second epitope, we selected a protective CD4+ T cell epitope with the idea that the combination of humoral and cell-mediated immune responses could strengthen the overall anticancer effect of immunization. The data indicate that immunization with OMVs engineered with the B cell epitope strongly protected mice from tumor challenge and that 100% protection was achieved with OMVs decorated with both the B and the T cell epitopes.

RESULTS

Selection of Cancer Antigens and Mouse Model

Since our first objective was to test whether the OMV-based vaccine platform could induce protective immune responses

in a cancer model of immunocompetent mice, we focused our attention on C57BL/6-B16F10 model and we selected two peptide antigens, LEEKKGNYVVTDH (EGFRvIIIpep) and PSKPSFQEFVDWENVSPELNSTDQPFL (B16-M30pep), previously shown to be protective in the same model.

EGFRvIIIpep belongs to EGFRvIII, a mutated form of the human epidermal growth factor receptor (EGFR), expressed on several tumors and associated with the expression of epithelial–mesenchymal transition and cancer stem cell genes. EGFRvIII contains an in-frame deletion in the extracellular domain of EGFR, creating a novel antigenic epitope which is exquisitely tumor-specific (27). Immunization with EGFRvIIIpep conjugated to limpet hemocyanin (KLH) was shown to protect mice from the challenge of syngeneic cell lines stably transfected with human EGFRvIII. In particular, Heimberger and coworkers showed that the conjugated peptide formulated with GM-CSF protected C57BL/6 mice from both extracerebral and intracerebral challenge with B16F10-EGFRvIII cells (28). Based on these data, a vaccine (Rindopepimut) for EGFRvIII-positive glioblastoma patients was proposed and tested in different trials (29). As far as the B16-M30pep is concerned, it was recently described by Kreiter and coworkers (7) as a CD4+ T cells epitope expressed in the B16F10 cell line as a consequence of a mutation occurred in the *kif18b* gene. Therefore, M30 is a B16F10-specific neo-epitope not expressed in the syngeneic healthy C57BL/6 mouse tissues. Interestingly, the authors showed that immunization with liposome-formulated synthetic RNA coding for B16-M30 induced robust T cell-mediated protection in C57BL/6 mice when challenged with B16F10 cells.

Immunogenicity and Protective Activity of EGFRvIII-OMVs

We first tested whether OMVs decorated with the Nm-fHbp-vIII fusion protein carrying three copies of EGFRvIIIpep at its C-terminus could induce anti-EGFRvIIIpep antibodies and whether such anti-EGFRvIIIpep immune response could protect mice from B16F10EGFRvIII challenge. The expression of EGFRvIIIpep in the OMVs from *E. coli* BL21Δ*ompA* strain has been recently described (20). Briefly, a synthetic DNA encoding three copies of EGFRvIIIpep was fused to the 3′ end of the *Neisseria meningitidis* fHbp gene, thus generating a chimera (Nm-fHbp-vIII) constituted of the full length fHbp protein and the EGFRvIII tri-peptide attached to its C-terminus (**Figure 1A**). The fusion protein was shown to be incorporated into the outer membrane of *E. coli* BL21Δ*ompA* and importantly to be exposed on the cell surface (**Figures 1C,D**). Furthermore, Nm-fHbp-vIII accumulates in the vesicle compartment, as demonstrated by SDS-PAGE and Western Blot analyses (**Figure 1B**) and by immune gold transmission electron microscopy (TEM) analysis of OMVs (**Figure 1E**).

C57BL/6 mice (16 mice per group) were immunized with either "empty" OMVs (not carrying the fused antigen) from *E. coli* BL21Δ*ompA* (control group) or with Nm-fHbp-vIII-OMVs. Vaccination was carried out at days 0, 14, and 28 (**Figure 2A**) and 1 week after the third immunization sera were collected and the induction of anti-EGFRvIII-antibodies was confirmed by ELISA (**Figure 2B**). A good fraction of EGFRvIII-specific antibodies belonged to the IgG2a isotype, in line with our previous data showing that OMVs from *E. coli* BL21Δ*ompA* elicit a Th1-skewed immune response (19). Next, at day 35, mice were challenged with a s.c. injection of 0.5×10^5 B16F10EGFRvIII cells and tumor growth was followed both in control mice and in mice immunized with Nm-fHbp-vIII-OMVs. While all but one control mice developed large tumors 20 days after challenge (average tumor volume = 850 mm^3, with three mice sacrificed having developed tumors >1,500 mm^3), immunization with Nm-fHbp-vIII-OMVs markedly reduced tumor growth in a statistically significant manner. In particular, eight mice were completely protected while the remaining mice developed tumors with average volumes of approximately 400 mm^3 (**Figure 2C**).

We also analyzed the tumor-infiltrating cell population in both control and Nm-fHbp-vIII-OMVs immunized mice. At the end of the challenge study, two tumors per group were randomly collected. Cells were mechanically and enzymatically isolated and the fraction of CD4+ T cells, CD8+ T cells, Treg, and MDSCs populations was determined by flow cytometry analysis after cell staining with specific antibodies. As shown in **Figure 2D** (Figure S1 in Supplementary Material), in line with the Th1 profile of the immune response, Nm-fHbp-vIII-OMVs immunization promoted a significant increase of CD4+ and CD8+ T cells at tumor site and a concomitant reduction of both CD4+ Treg and MDSC cells.

Synergistic Protective Activity of EGFRvIIIpep and M30

Having demonstrated that EGFRvIII-OMVs induced a robust protection in C57BL/6 mice challenged with B16F10EGFRvIII cell line, we investigated whether protection could be further potentiated by formulating Nm-fHbp-OMVs with B16-M30pep, a second antigen expressed in B16F10EGFRvIII and generated by one of the several B16F10-specific mutations (7). Therefore, we set up a second immunization/challenge experiment involving four groups of eight mice each. The first group received three doses of "empty" OMVs from *E. coli* BL21Δ*ompA* (control group). The second group was injected with "empty" OMVs together with B16-M30 synthetic peptide (100 μg/dose) (peptide-"absorbed" M30-OMVs). Finally, the third and the fourth groups received three doses of Nm-fHbp-vIII-OMVs and three doses of Nm-fHbp-vIII-OMVs mixed with B16-M30pep, respectively (peptide-"absorbed" M30-Nm-fHbp-vIII-OMVs). One week after the last immunization, all mice were challenged with 0.5×10^5 B16F10EGFRvIII cells and tumor growth was followed over a period of 30 days. **Figure 3** summarizes the result of this experiment. In line with the previous experiment, EGFRvIII-OMVs induced a strong protective immunity against B16F10EGFRvIII. Five out of eight mice were completely protected and the other three mice developed tumors with an average size of approximately 350 mm^3. All but one control mice developed tumors ≥1,500 mm^3 and were euthanized. As far as M30-"absorbed" vesicles are concerned, M30-OMVs immunization resulted in a marginal, non-statistically significant protection, with only two out of eight mice protected. However, when B16-M30 peptide

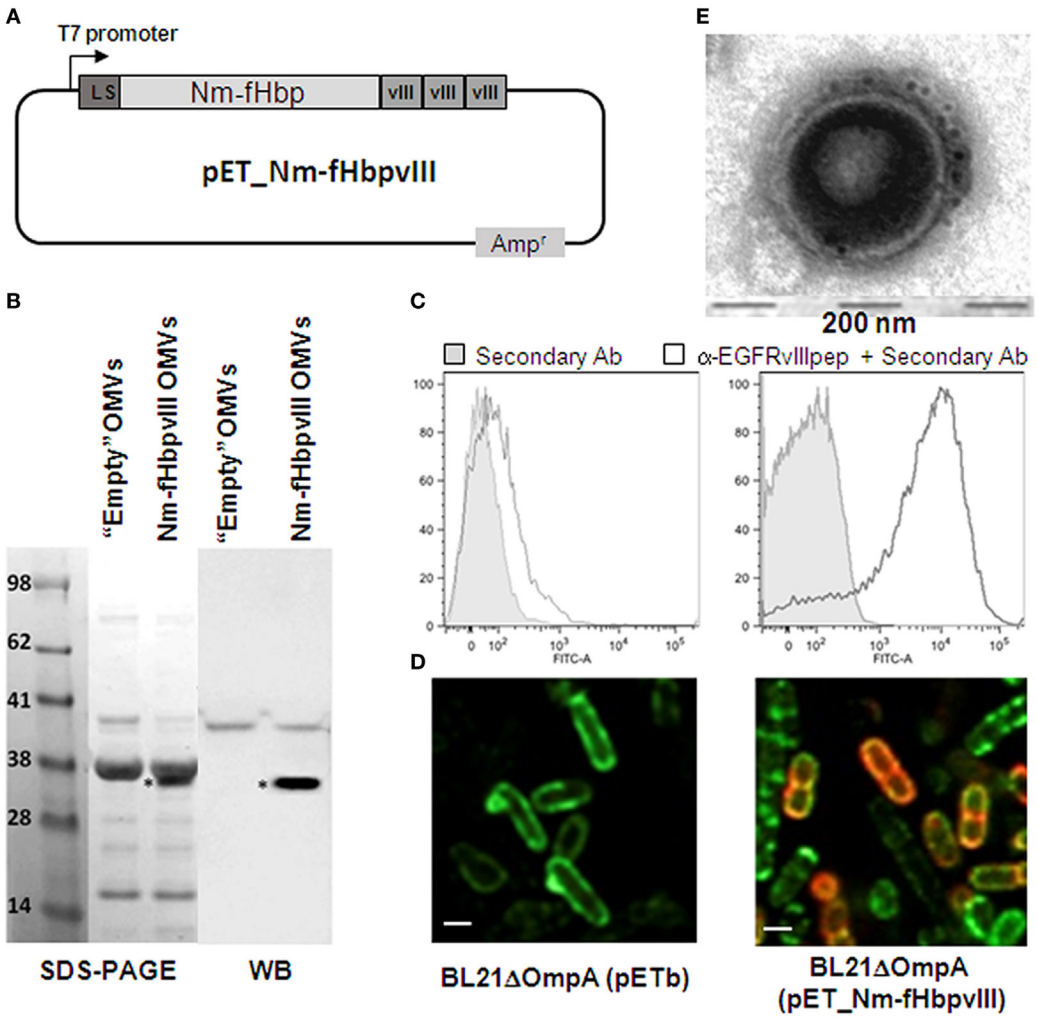

FIGURE 1 | Expression and surface localization of EGFRvIII epitope in BL21Δ*ompA*(pET-Nm-fHbpvIII) strain and in its derived outer membrane vesicles (OMVs). **(A)** Schematic representation of pET-Nm-fHbpvIII plasmid encoding three copies of EGFRvIIIpep fused to the C-terminus of *Neisseria meningitidis* fHbp. **(B)** SDS-PAGE and Western Blot analyses of OMVs. OMVs were purified from BL21Δ*ompA*(pET21b+) ("Empty" OMVs) and BL21Δ*ompA*(pET-Nm-fHbpvIII) strains and loaded on SDS-polyacrylamide gels for SDS-PAGE analysis (20 μg OMVs) and Western Blot analysis (1 μg OMVs). After proteins transfer to the nitrocellulose membrane, Nm-fHbp-vIII fusion was visualized using rabbit anti-EGFRvIIIpep antibodies and peroxidase-conjugated anti-rabbit immunoglobulins. **(C)** Flow cytometry analysis of BL21Δ*ompA*(pET21b+) and BL21Δ*ompA*(pET-Nm-fHbpvIII) strains. Bacterial cells were incubated first with anti-EGFRvIIIpep rabbit antibodies and subsequently with FITC-labeled anti-rabbit secondary antibodies. Fluorescence was measured by flow cytometry. Gray areas represent the background fluorescence signals obtained incubating the cells with the secondary antibody only. **(D)** Confocal microscopy analysis of BL21Δ*ompA*(pET21b+) ("Empty" OMVs) and BL21Δ*ompA*(pET-Nm-fHbpvIII) strains. After induction of protein expression with IPTG, bacterial cells were fixed in 4% formaldehyde solution and incubated first with rabbit anti-EGFRvIIIpep polyclonal antibodies and mouse anti-LPS mAb, and subsequently with goat anti-rabbit IgG, Alexa Fluor 594 conjugated-antibodies (red), and goat anti-mouse IgG, Alexa Fluor 488 conjugated-antibodies (green). **(E)** Immuno Transmission Electron Microscopy (TEM) analysis of OMVs purified from BL21Δ*ompA*(pET-Nm-fHbpvIII) strain using primary anti-EGFRvIIIpep rabbit antibodies and 5-nm gold-labeled anti-rabbit secondary antibody (see Materials and Methods for details).

was "absorbed" to Nm-fHbp-vIII-OMVs, protection from tumor growth was complete, with only one mouse scored as having a "barely detectable tumor" (**Figure 3A**).

The conclusion from these experiments is that the M30 peptide "absorbed" to "Empty" OMVs induced an M30-specific immune response not sufficient to protect mice from the challenge with B16F10EGFRvIII cell line, but capable of synergizing with a second antigen (a B cell epitope) to the point that together the two antigens completely abrogated tumor growth.

To evaluate the immunogenicity of the M30 peptide "absorbed" to OMVs, the presence of M30-specific, IFN-γ-positive T cells was analyzed in the spleens of mice sacrificed at the end of the challenge experiment. As shown in **Figure 3B**, mice immunized with both M30-OMVs and M30-EGFRvIII-OMVs had a higher number of M30-specific, CD4+ T cells with respect to spleens of control-group mice. By contrast, no appreciable amounts of M30-specific CD8+ T cells were measured (not shown), in line with the fact that M30 was described as a MHC II neo-epitope (7).

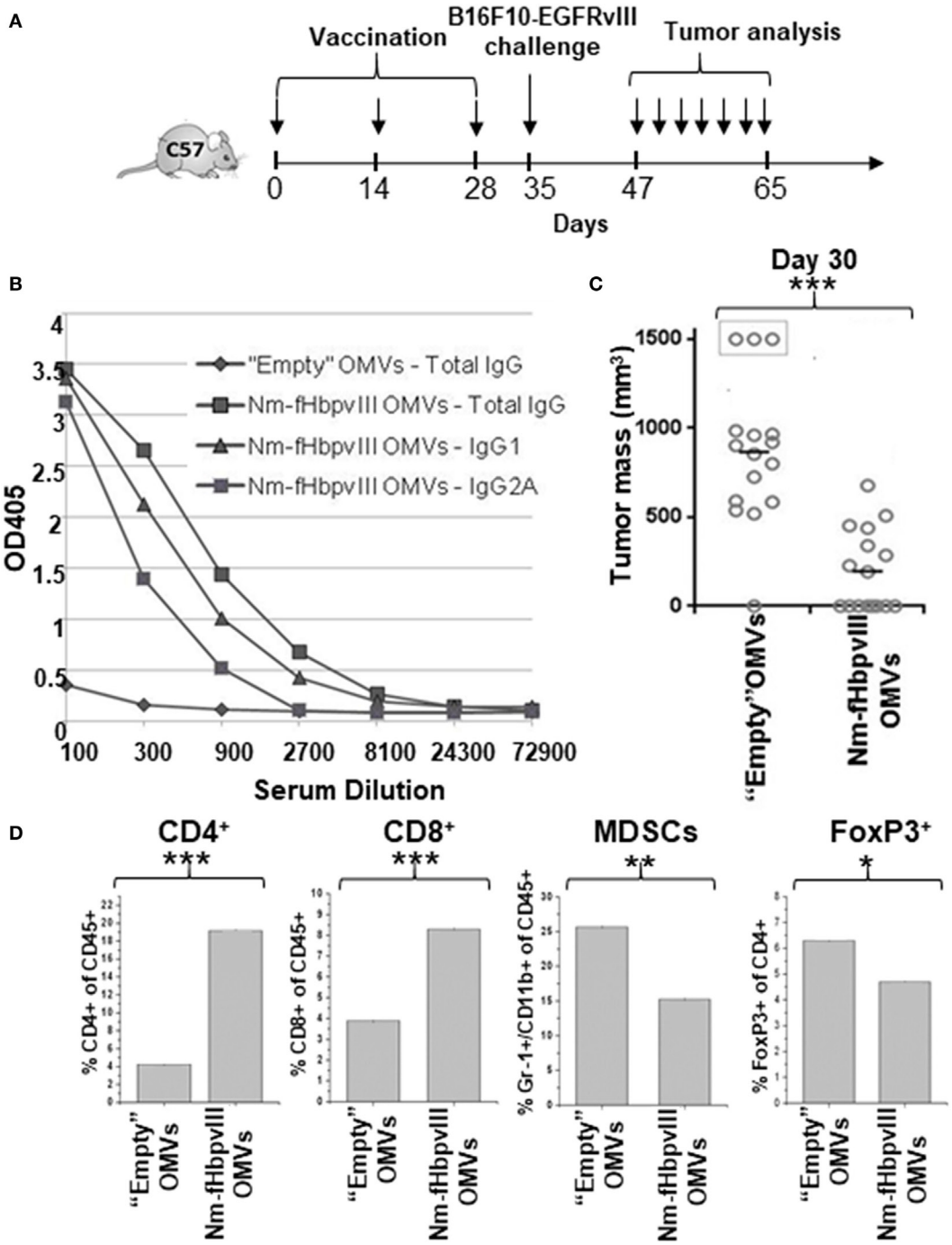

FIGURE 2 | Immunogenicity and protective activity of Nm-fHbpvIII-outer membrane vesicles (OMVs). **(A)** Schematic representation of immunization and challenge schedules in C57BL/6 mice. **(B)** Anti-EGFRvIIIpep antibody titers in C57BL/6 mice immunized with "Empty" OMVs and with Nm-fHbpvIII-OMVs. Sera from mice immunized as reported in **(A)** were pooled and total IgGs, IgG1, and IgG2a were measured by ELISA, coating the plates with synthetic EGFRvIIIpep (0.5 μg/well). **(C)** Analysis of tumor development in C57BL/6 mice immunized with "Empty" OMVs and with Nm-fHbpvIII-OMVs. The figure reports the tumor size in each mouse as measured at day 30 after challenge with 0.5×10^5 B16F10EGFRvIII cells. *** indicates a statistically significant difference of $P < 0.001$. **(D)** Analysis of tumor-infiltrating cell populations. At the end of the challenge experiment, two tumors/group were randomly selected and the percentage of infiltrating CD4+ T cells, CD8+ T cells, MDSCs, and Tregs was determined by flow cytometry, as described in Section "Materials and Methods" (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Finally, to further confirm the synergistic effect of EGFRvIIIpep and M30 in protecting mice from B16F10EGFRvIII cell line challenge, we created a second fusion protein in which fHbp was fused to three copies of M30 peptide followed by three copies of EGFRvIII pep (**Figure 4A**). The construction details of plasmid pET-Nm-fHbp-M30-vIII encoding the fusion protein

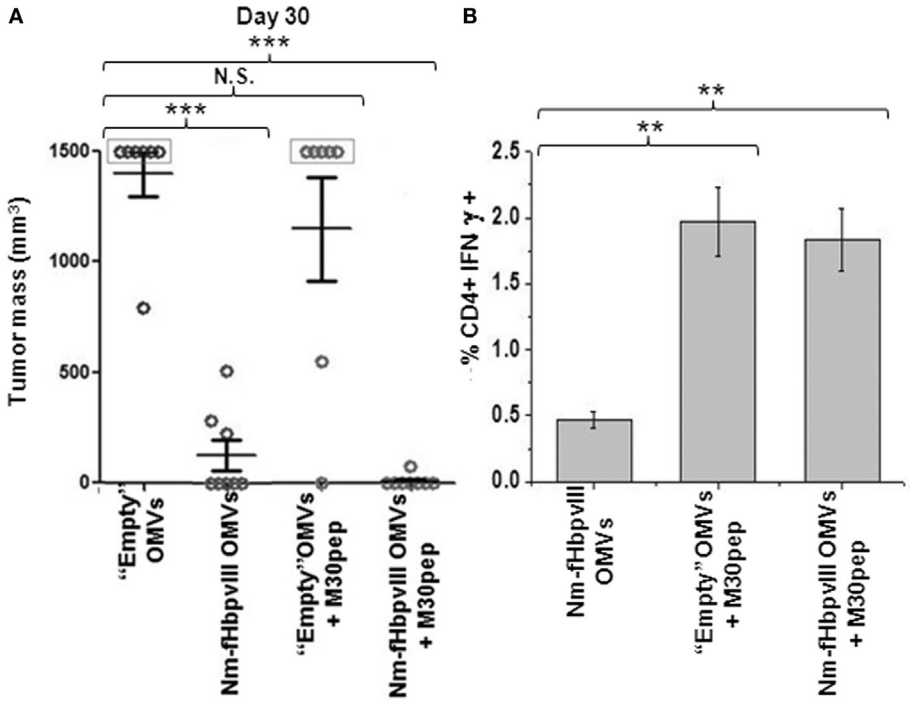

FIGURE 3 | Synergistic protective activity of Nm-fHbpvIII-OMVs/M30 peptide combination. **(A)** Analysis of tumor development in C57BL/6 mice immunized as shown in **Figure 2A**. The figure reports the tumor size in each mouse as measured at day 30 after the challenge with 0.5×10^5 B16F10EGFRvIII cells. *** indicates that the difference in tumor size between each group and control group is statistically significant with $P < 0.001$. **(B)** Analysis of M30pep-specific CD4+ T cells in immunized mice. At the end of the challenge experiment, spleens from two animals were collected. Splenocytes were stimulated with M30pep and IFNγ-producing CD4+ T cells were analyzed by flow cytometry (**$P < 0.01$).

are reported in the Section "Materials and Methods." The fusion protein accumulated in the OMV compartment and the engineered OMVs induced anti-EGFRvIII antibody titers similar to the titers induced by Nm-fHbp-vIII-OMVs (**Figure 4B**). Moreover, Nm-fHbp-M30-vIII-OMVs induced IFN-γ positive, M30-specific, CD4+ T cells to a level comparable to the induction observed upon immunization with M30—"absorbed" OMVs (100 μg M30pep + 20 μg OMVs) (**Figure 4C**). Finally, when mice immunized with Nm-fHbp-M30-vIII-OMVs were challenge with the B16F10EGFRvIII cell line, all animals were completely protected with no sign of tumor development at the site of injection (**Figure 4B**).

DISCUSSION

This work delivers a few relevant messages.

First of all, we have shown that the OMV vaccine platform can be potentially applicable in cancer immunotherapy. OMVs are being extensively and successfully utilized in the preclinical and clinical settings for prophylactic vaccination against infectious diseases [for a recent review, see Ref. (30)]. Their unique adjuvanticity, which directs the immune responses toward a marked Th1 profile, and the ease with which they can be manipulated and purified have attracted the attention of several academic and industrial groups and bacterial OMV-based vaccines are already available for human use. However, there is a paucity of information regarding the applicability of this platform technology in cancer vaccines. Our data demonstrate that OMVs are a promising alternative to other adjuvants/delivery systems. EGFRvIII-decorated vesicles are capable of inducing a potent anti-EGFRvIII antibody response which, in immune competent C57BL/6 mice, strongly reduced the growth of B16F10 tumor cells expressing human EGFRvIII. Moreover, the Th1 profile of the response favored the migration of IFNγ-producing CD4+ and CD8+ T cells at the tumor site, eventually contributing to the overall protective activity of vaccination. The level of protection obtained appears to be similar to the one described by Heimberger and coworkers using the same mouse model and a KLM-conjugated EGFRvIII peptide in the presence of GM-CSF (28).

A second important message from this work is that the decoration of OMVs with more than one antigen further potentiate the protective efficacy of the vaccine. In particular, we combined a B cell epitope to a CD4+ T cell epitope and we showed that, together, the two epitopes completely abrogate tumor growth. This is an interesting observation also in light of the fact that in glioblastoma patients, vaccination with EGFRvIII-conjugated peptide was shown to prolong overall survival but ultimately EGFRvIII-negative tumor cells escape vaccine-induced protection (29). This immunoediting mechanism can in part explain the disappointing results obtained with the EGFRvIII-conjugated vaccine in a large Phase III trial (31). Our data pointing to the synergistic effect of EGFRvIII-OMV in combination with other cancer-specific epitopes might rejuvenate the interest in EGFRvIII antigen in the near future.

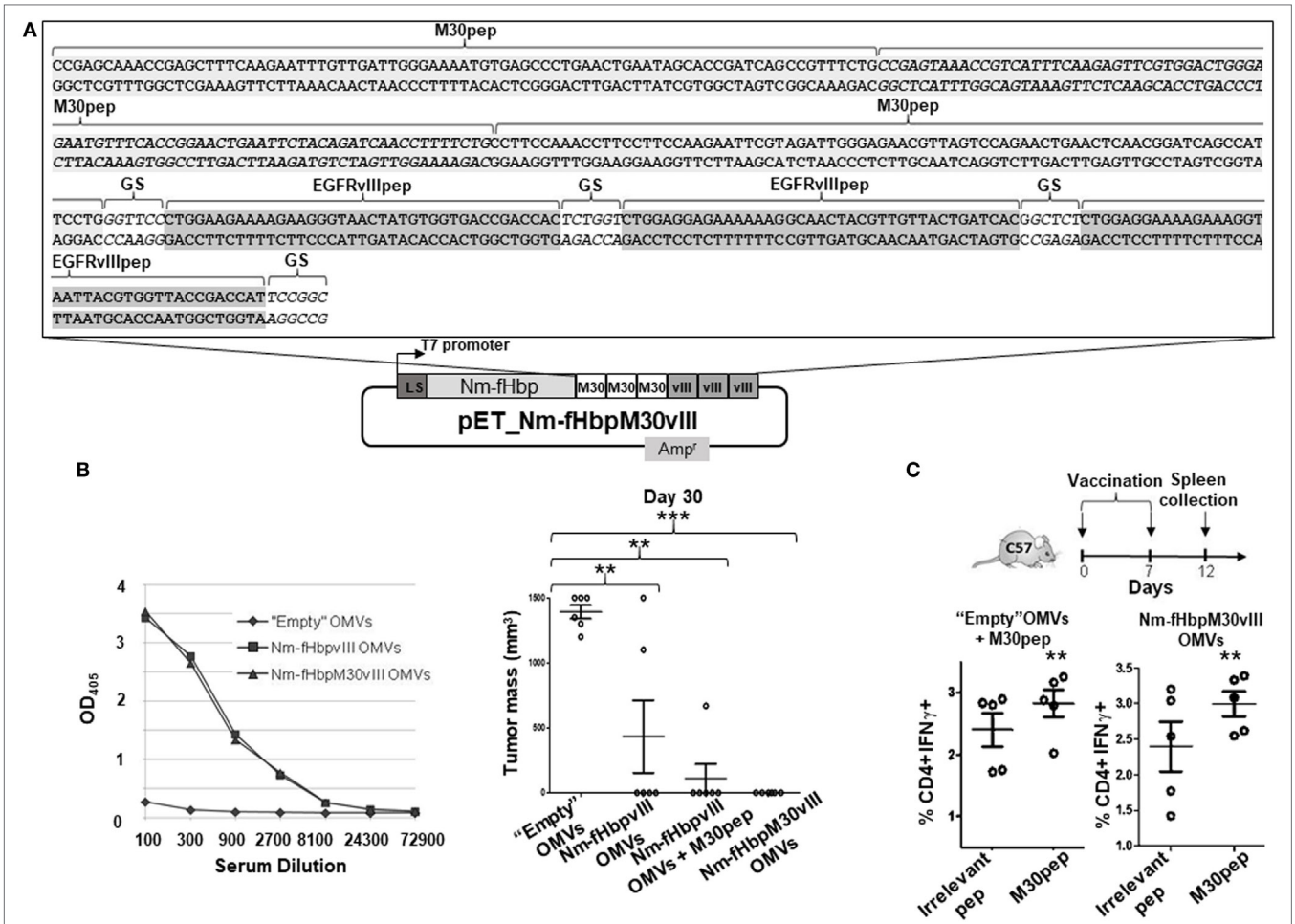

FIGURE 4 | Immunogenicity and protective activity of outer membrane vesicles (OMVs) decorated with Nm-fHbp-M30-vIII fusion. **(A)** Schematic representation of pET-Nm-fHbp-M30-vIII plasmid. The DNA sequence refers to the 3′ end of the gene fusion encoding three copies of both M30 and EGFRvIII epitopes. **(B)** Mice were immunized with (i) "Empty" OMVs, (ii) Nm-fHbpvIII OMVs, and (iii) Nm-fHbpvIII OMVs + M30pep, and (iv) Nm-fHbp-M30-vIII-OMVs and subsequently challenged with 0.5×10^5 B16F10EGFRvIII cells following the schedule indicated in **Figure 2A**. Tumor size in each mouse was measured at day 30 post challenge. Seven days after the last immunization, serum samples were also collected from mice immunized with (i) "Empty" OMVs, (ii) Nm-fHbpvIII OMVs, and (iii) Nm-fHbp-M30-vIII-OMVs, and total anti-EGFRvIII IgGs were measured by ELISA (**$P < 0.01$; ***$P < 0.001$). **(C)** M30-specific CD4+ T cells induced in mice immunized with OMVs decorated with M30 peptide. Mice were immunized twice i.p. at days 0 and 7 with either 20 μg "Empty" OMVs + 100 mg M30pep or 20 μg Nm-fHbp-M30-vIII-OMVs. Five days after the second immunization, splenocytes were stimulated with either an irrelevant peptide or with M30 peptide and IFNγ-positive CD4+ T cells were counted by FACS (**$P < 0.01$).

A third message from our work is the confirmation that the OMV platform can efficiently elicit not only humoral but also cell-mediated immunity against OMV-associated heterologous antigens. Even though the elicitation of protective T cell responses using pathogen-derived whole vesicles (32) or using OMVs decorated with heterologous antigens (33) was described, information on the general applicability of the OMV platform to induce antigen-specific cell-mediated immunity is still limited. Our work further provides evidence that OMVs combined or engineered with new T cell epitopes elicit epitope-specific T cell responses. We recently corroborated this conclusion by engineering OMVs with seven additional cancer CD4+/CD8+ T cell epitopes and by demonstrating the induction by all seven engineered OMVs of epitope-specific T cell responses (manuscript in preparation). Considering the ease with which OMVs can be manipulated with foreign antigens, these results lead to the attractive possibility of exploiting the OMV platform in cancer precision medicine.

One last comment deserves the strength of T cell responses induced by OMVs. Kreiter and coworkers previously shown that the M30 CD4+ T cell epitope completely inhibited tumor growth using a mouse model similar to the one tested in this study. Furthermore, the same authors showed that M30 immunization could also reduce the formation of lung metastases in the same model (7). In our hands, protection mediated by M30-"absorbed" OMV immunization could only be appreciated in combination with EGFRvIII epitope. There are a number of arguments to explain the different results. First, differently from the data reported by Kreiter and coworkers (7), we used a "classical" prophylactic modality according to which three

immunizations were followed by the tumor challenge. While this schedule is indicated for eliciting antibody responses, it is not typically recommended for T cell responses, which usually require several administrations few days apart. Second, the i.v. route of immunization used by Kreiter and coworkers appears to be a key element to obtain the remarkable protection of M30 peptide. By delivering the vaccine intravenously, these authors showed that the vaccine could reach the spleen where it could be taken up by dendritic and phagocytic cells. Third, Kreiter and coworkers used as vaccine synthetic RNA coding for the M30 peptide. RNA vaccines have the property to drive the expression of the antigen directly into the cytoplasm of receiving cells and to act as potent adjuvant. While the armamentarium of adjuvants present in OMVs, which work through the elicitation of several TLR and NOD signaling pathways, should guarantee excellent Th1 immune responses, we are currently testing whether different immunization schedules and routes of immunization might improve the level of protection of M30-formulated OMVs. In this respect, we recently challenged BALB/c mice with CT26 cell line, and after challenge, mice were given seven immunizations 3 days apart with OMVs decorated with five protective CT26 neoeptopes described by Kreiter et al. (7). Following this therapeutic immunization modality tumor growth was remarkably reduced (manuscript in preparation). Finally, it has to be pointed out that when tested alone in our immunization/challenge experiments M30 peptide was "absorbed" to OMVs. In reality, we do not know the interaction of the M30 peptide to the OMVs and in fact, considering the hydrophobic nature of several amino acids and the presence of a few negatively charged amino acids, the peptide might not stably interact with the vesicles at all. Should this be the case, since adjuvant/antigen co-delivery to DCs is a pre-requisite to elicit good T cell responses, M30-engineered OMVs should outperform the M30-"absorbed" OMVs. We have not tested yet the protective activity of M30-engineered OMVs but it is interesting to note that Nm-fHbp-M30-vIII-OMVs fusion induced good levels of M30-specific, CD4+ T cells and fully protected mice from tumor challenge even if, on a molar basis, the amount of M30 peptide present in the engineered OMVs was approximately 1,000-folds lower than the 100 μg theoretically "absorbed" to the OMVs. In fact, assuming that the fusion protein represents 2–5% of total OMV proteins (**Figure 4B**), each mouse received approximately 0.5–1 μg of fusion protein/vaccine dose, corresponding to no more than 50–100 ng of M30 peptide.

In conclusion, our work demonstrates that bacterial OMVs represent a promising platform for cancer immunotherapy. The main interesting aspects of the technology includes (i) the rapidity with which they can be decorated with foreign epitopes (we routinely engineer the OMV-producing strains with heterologous antigens in less than 2 weeks), (ii) the high yield of OMVs from bacterial fermentation (usually more than 100 mg of purified OMVs are obtained from a 1-l fermentation), and (iii) the simplicity of the OMV purification process, which only involves tangential flow ultrafiltration. Considering that OMVs are already part of specific human vaccines for which the safety and the quality control assays have already been developed, the platform is potentially ready to be tested in the clinics.

MATERIALS AND METHODS

Bacterial Strains, Cell Line, and Mice

Escherichia coli HK100 strain was used for cloning experiments using the PIPE method.

B16F10 melanoma cell line that stably expresses the EGFRvIII variant gene was kindly provided by Prof. Sampson (Department of Neurosurgery of the Duke University, Duhram, NC, USA). Cells were tested for mycoplasma before animal injection.

To verify the presence of the M30-associated mutation in B16F10 cell line, RNA from B16F10 cells was purified using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Subsequently, purified RNA was reverse-transcribed to cDNA using qScript cDNA synthesis kit (Quanta Biosciences). Finally, the region spanning the M30-associated mutation was PCR amplified from B16F10 cDNA with the forward (TCCTCCCGAGTCTGCCCAGCCACGGTCATT) and the reverse (ACAGCTGCGGCCTCGGGAGACTGAGGGCCT) primers. The amplification reaction product was purified from agarose gel using the PCR clean-up Kit (Macherey Nagel) and sequenced.

C57bl/6 female 4-week-old mice were purchased from Charles River Laboratories and kept and treated in accordance with the Italian policies on animal research at the Toscana Life Sciences animal facility (Siena, Italy).

Construction of Plasmids

The construction of pET21-Nm-fHbp and pET-Nm-fHbp-vIII plasmids expressing the *Neisseria meningitidis* fHbp and fHbp fused to three repeated copies of EGFRvIII peptide, respectively, was previously described (20). pET-Nm-fHbp-M30vIII plasmid carries the *N. meningitidis fHbp* gene fused to a synthetic DNA fragment encoding three copies of B16-M30 peptide and three copies of EGFRvIII peptide, each copy intercalated by a Glycine–Serine (GS) spacer (**Figure 4A**). To construct the plasmid, the PIPE method was applied. Briefly, pET-Nm-fHbp-vIII plasmid was linearized by PCR, using F-vIIIM30 (5′-ATCAGCCATTCCTGGGTTCCC TGGAAGAAAAGAAGGGT-3′) primer, which anneals upstream of the vIII coding sequence, and R-fHbpM30(5′-TGCCTAGT CGGTAAGGACTTATTGCTTGGCGGCAAGGC-3′) primer. In parallel, the synthetic DNA encoding three copies of M30 peptide (Thermo Fisher, 1 ng/μl in MilliQ water) was amplified by PCR with the forward primer 5′-CTTGCCGCCAAGCAAC CGAGCAAACCGAGCT-3′, complementary to the 5′ end of the *N. meningitidis fHbp* gene, and the reverse primer 5′-TCT TCCAGGGAACCCAGGAATGGCTGATCCGTTGA-3′ complementary to the vIII sequence and encoding a GS spacer. The PCR products were mixed together and the mixture was used to transform *E. coli* HK100 strain. After confirmation of the correctness of the gene fusion by sequence analysis, *E. coli* BL21DE3*ΔompA* strain was transformed with pET-Nm-fHbp-M30-vIII plasmid and the derived recombinant strain was used for the production of engineered M30-vIII-OMVs.

Synthetic Peptides and Antibodies

The EGFRvIII peptide LEEKKGNYVVTDH unconjugated or conjugated to KLH protein was purchased from GeneScript in

lyophilic form and solubilized in PBS at the final concentration of 1 mg/ml. Polyclonal antibodies against EGFRvIII peptide were obtained from GenScript by immunizing rabbits with KLH-conjugated LEEKKGNYVVTDH peptide.

The 27 amino acid M30 peptide PSKPSFQEFVDWEN VSPELNSTDQPFL was purchased from GeneScript in lyophilic form and solubilized in milliQ water at final concentration of 5 mg/ml.

Bacterial Total Lysate and OMV Preparation

Plasmids containing the genes of interest were used to transform *E. coli* BL21DE3Δ*ompA* strain. Recombinant clones were grown in 200 ml LB medium (starting OD_{600} = 0.05) and, when the cultures reached an OD_{600} value of 0.5, protein expression was induced by addition of 1 mM IPTG. After 2 h, OMVs were collected from culture supernatants by filtration through a 0.22-μm pore size filter (Millipore) followed by high-speed centrifugation (200,000 *g* for 2 h). Pellets containing OMVs were finally re-suspended in PBS. Total bacterial lysates were prepared by suspending bacterial cells from 1 ml cultures (centrifuged at 13,000 *g* for 5 min) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Laemli buffer and heated at 100°C for 5 min. Proteins were separated by 4–12% or 10% SDS-PAGE (Invitrogen), run in MES buffer (Invitrogen), and finally stained with Coomassie Blue.

Western Blot Analysis

Total lysates were prepared from bacteria grown in LB. Liquid cultures were pelleted in a bench-top centrifuge and suspended in SDS-PAGE loading buffer in an appropriate volume to normalize cell density to a final OD_{600} = 10. Each sample (10 μl) was then separated on a 4–12% SDS-polyacrylamide gel (Invitrogen). Proteins separated by SDS-PAGE were then transferred onto nitrocellulose membrane by standard methods. The membranes were blocked either 1 h at room temperature (RT) or overnight at 4°C by agitation in blocking solution (10% skimmed dry milk and 0.05% Tween 20 dissolved in PBS). Primary antibodies or sera were diluted in 1% skimmed dry milk plus 0.05% Tween 20 dissolved in PBS and incubated 1 h at RT. After three washing steps in 0.05% Tween 20 dissolved in PBS, the membranes were incubated in a 1:2,000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Dako) in 1% skimmed dry milk and 0.05% Tween 20 dissolved in PBS for 1 h, and after three washing steps, antibody binding was detected by using the SuperSignal West Pico chemiluminescent substrate (Pierce).

Flow Cytometry Analysis

20 ml of LB medium supplemented with 100 μg/ml Ampicillin were inoculated at OD_{600} = 0.05 with an overnight culture of BL21Δ*ompA*(pET-Nm-fHbp-vIII). The culture was then grown and IPTG-induced as described above. BL21Δ*ompA*(pET21b+) strain was used as negative control. Bacterial cells from 1 ml were harvested by centrifugation at 10,000 *g* for 5 min at 4°C and re-suspended with 1% BSA in PBS to obtain a cell density of 2×10^7 CFUs/ml. 50 μl were then dispensed in a round bottom 96 well plate. Anti-EGFRvIIII peptide rabbit antibodies were added at a concentration of 5 μg/ml and incubated 1 h on ice. After three washes with 1% BSA in PBS, 20 μl of FITC-labeled anti-rabbit secondary antibodies (1:200 dilution) (Life Technologies) were added and incubated 30 min on ice. Each well was then washed twice with 200 μl 1% BSA in PBS, and plates were centrifuged at 4,000 *g* for 5 min. Samples were then re-suspended in 2% formaldehyde solution, incubated 15 min at 4°C and centrifuged again at 4,000 *g* for 5 min. Finally, samples were re-suspended in 200 μl of PBS, and data were acquired by using BD FACS Canto II cell analyzer (BD).

Confocal Microscopy Analysis

To verify fHbp-EGFRvIII localization on the cell surface, 20 ml of LB medium were inoculated at OD_{600} = 0.05 with an overnight culture of BL21Δ*ompA*(pET-fHbpvIII). The culture was grown and protein expression induced with IPTG as described above. Bacterial cells from 1 ml culture were harvested by centrifugation at 6,000 *g* for 5 min at 4°C and re-suspended in 4% formaldehyde solution, incubated 15 min at 4°C and then centrifuged at 6,000 *g* for 5 min. Then samples were washed three times with 1 ml PBS, and incubated in 1 ml of blocking buffer (0.1% BSA, 10% normal goat serum in PBS) 20 min at RT. Subsequently, the bacterial suspension was incubated with rabbit polyclonal anti-EGFRvIIIpep antibodies (1 μg/ml) and mouse anti-LPS mAb (1 μg/ml) (Hycult Biotech, USA), for 1 h at RT. After two washes with 0.1% BSA in PBS, bacteria were incubated for 20 min at RT with goat anti-rabbit IgG, Alexa Fluor 594 conjugated-antibodies (Molecular Probes) and goat anti-mouse IgG, Alexa Fluor 488 conjugated-antibodies (Molecular Probes) at 1:400 final dilution. Labeled bacteria were washed twice with 0.1% BSA in PBS, and allowed to adhere to poly-lysine slides (Thermo Scientific) for 20 min at RT. Slides were mounted with ProLong Gold antifade reagent (Thermo Scientific). Confocal microscopy analysis was performed with a Leica SP5 microscope and images were obtained using Leica LASAF.

TEM Analysis

Outer membrane vesicles purified from *E. coli* BL21Δ*ompA*(pET-Nm-fHbpvIII) strain were visualized using Immuno TEM. Briefly, a 5-μl aliquot of purified OMVs preparation at a final concentration of 20 ng/μl was applied to 200-square mesh nickel grids coated with a thin carbon film (Agar Scientific) and let stand for 3 min. The samples were then blocked in 0.5% BSA in PBS for 1 h at RT. Subsequently, the samples were incubated with primary rabbit anti-EGFRvIIIpep antibodies for 1 h at RT. Grids were washed three times in blocking buffer and incubated with 5-nm gold-labeled anti-rabbit secondary antibody (BB International, Madison, WI, USA) for 1 h at RT. Immunostained OMVs were then negatively stained in 1% phosphotungstic acid and visualized with a Tecnai G2 Spirit Transmission Electron Microscope operating at 100 kV. Images were collected at 87,000× magnification with a CCD camera Morada 2kx4k.

Vaccine Immunogenicity and Tumor Challenge in C57BL/6 Mice

Immunization with OMVs from BL21*ΔompA* (pET-Nm-fHbp-vIII) Strain

C57BL/6 mice (16 mice/group) were vaccinated on day 0, 14, and 28 with 20 μg of either "empty" OMVs [derived from BL21*ΔompA* (pET21b+) strain] or 20 μg of Nm-fHbpvIII-OMVs [derived from BL21*ΔompA*(pET-Nm-fHbp-vIII) strain] formulated in PBS. At day 35, 0.5×10^5 B16F10EGFRvIII cells were subcutaneously (s.c.) injected in each animal and tumor growth was measured with a caliper every 3 days over a period of 30 days. For ethical reasons, mice were euthanized when tumors reached a size of 1,500 mm^3.

Immunization with Nm-fHbp-vIII-OMVs Combined with B16-M30 Peptide

C57BL/6 mice (eight mice/group) were vaccinated on day 0, 14, and 28 with 20 μg "empty" OMVs, 20 μg Nm-fHbp-vIII-OMVs, 100 μg of synthetic B16-M30 peptide absorbed to 20 μg Nm-fHbp-vIII-OMVs, or 20 μg Nm-fHbp-M30-vIII-OMVs. At day 35, 0.5×10^5 B16F10EGFRvIII cells were s.c. injected in each animal and tumor growth was followed as descried above.

Analysis of Anti-EGFRvIII Antibodies in Immunized Animals

Anti-EGFRvIII antibodies were measured by ELISA. Amino plates (Thermo Fisher) were coated with synthetic EGFRvIII peptide (0.5 μg/well) and incubated overnight at 4°C. The day after, plates were saturated with a solution of 1% BSA in PBS (200 μl per well) for 1 h at 37°C. Mice sera were threefold serially diluted in PBS supplemented with 0.05% tween (PBST) and 0.1% BSA. After three washes with PBST, 100 μl of each serum dilution were dispensed in plate wells. As positive control, Anti-EGFRvIII rabbit serum from animals immunized with KLH-conjugated LEEKKGNYVVTDH (EGFRvIII) peptide was used. After 2 h incubation at 37°C, wells were washed three times with PBST and then incubated 30 min at 37°C with mouse anti-rabbit alkaline phosphatase-conjugate antibodies at a final dilution of 1:2,000. After three washes with PBST, 100 μl of alkaline phosphatase substrate (Sigma Aldrich) were added to each well and plates were maintained at RT in the dark for 30 min. Finally absorbance was read at 405 nm using the M2 Spectramax Reader plate instrument.

T Cell Analysis

At the end of the tumor challenge studies described above (30 days from tumor cell administration) mice were sacrificed and spleens collected in 5 ml DMEM high glucose (GIBCO). Alternatively, mice were immunized twice i.p. at days 0 and 7 with either 20 μg "Empty" OMVs + 100 mg M30pep or 20 μg Nm-fHbp-M30-vIII-OMVs. Five days after the second immunization, mice were sacrificed and spleens collected. Spleens were then homogenized and splenocytes filtered using a Cell Strainer 70 μm. After centrifugation at 400 *g* for 7 min, splenocytes were re-suspended in PBS and aliquoted in a 96-well plate at a concentration of 1×10^6 cells per well. Cells were stimulated with 2 mg/ml of an unrelated peptide (negative control), or 2 mg/ml of B16-M30 peptide. As positive control, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 0.5 mg/ml) and Ionomycin (1 mg/ml). After 2 h of stimulation at RT, Brefeldin A [Beckton Dickenson (BD)] was added to each well and cells incubated for 4 h at 37°C. After two washes with PBS, NearIRDead cell staining reaction mixture (Thermo Fisher) was incubated with the splenocytes for 20 min at RT in the dark. After two washes with PBS and permeabilization and fixing with Cytofix/Cytoperm (BD) using the manufacturer's protocol, splenocytes were stained with a mix of the following fluorescent-labeled antibodies: anti CD3-APC (BioLegend), anti-CD4-BV510 (BioLegend), anti-CD8-PECF594 (BD), and IFN-γ-BV785 (BioLegend). Samples were analyzed on a BD FACSCanto II using FlowJo software. Graphs were processed with Prism 5.0 software (Graphpad). Statistical analysis and differences in means between two groups were calculated with a *t*-test calculator carried out using GraphPad Prism 5.03 (n.s.: $P > 0.0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Analysis of TILs

Tumor-infiltrating lymphocytes were isolated from subcutaneous B16F10EGFRvIII tumors taken from sacrificed mice. At least two tumors per group were collected and minced into pieces of 1–2 mm of diameter using a sterile scalpel. Tumor samples were then transferred into 15-ml tubes containing 5 ml of collagenase solution (Collagenase Type 3,200 U/ml, Collagenase Type 4,200 U/ml) diluted in HBSS with 3 mM $CaCl_2$ and incubated under agitation for 2 h at 37°C. The resulting cell suspensions were filtered through a Cell Strainer 70 μm, washed twice with PBS and 1×10^6 cells were dispensed in a 96-well plate. Then, cells were incubated with NearIRDead cell staining Kit (Thermo Fisher) 20 min on ice in the dark. After two washes with PBS, samples were stained with the following mixture of fluorescent-labeled antibodies (BD): anti-GR1 (BV605), anti-CD11b-BV480, anti-CD45-BV786, anti-CD3-BV421, anti-CD4-PE, anti-CD8-PECF594, and anti-CD25-APC. The samples were then incubated 1 h at RT. After two washes with PBS, Cytofix/Cytoperm (BD) was added to each well and incubated 20 min on ice in the dark. After two washes with PBS, cells were stained with anti-Foxp3-A488 (BD) antibodies diluted in Permwash 1× buffer 20 min at RT in the dark. Finally, samples were washed two times with 1% BSA in PBS and analyzed on a BD FACSCanto II as described above.

ETHICS STATEMENT

Mice were monitored twice per day to evaluate early signs of pain and distress, such as respiration rate, posture, and loss of weight (more than 20%) according to humane end points. Animals showing such conditions were anesthetized and subsequently sacrificed in accordance with experimental protocols, which were reviewed and approved by the Animal Ethical Committee of Toscana Life Sciences Foundation and by the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

GG: design of experimental plan and manuscript preparation. AG: experimental design, strain Engineering, analysis of immune responses, and animal studies. MP: analysis of Immune responses, FACS analysis, T cell analysis, and confocal microsacopy analysis. MT and IZ: animal studies. LG, EC, LFantappiè, SI, FZ, and

CI: strain engineering and OMV preparation and characterization. LFagnocchi and EK: strain fermentation and OMV purification and characterization. ST and CS: FACS analysis of T cell population. FG and IF: electron microscopy analysis of OMVs.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fonc.2017.00253/full#supplementary-material.

FIGURE S1 | Flow cytometry gating strategy and analysis of tumor cell populations. Cells (1×10^6) were isolated after collagenase treatment from frozen tumors collected from one mouse immunized with "Empty" OMVs **(A)** and one mouse immunized with Nm-fHbpvIII OMVs **(B)**. A first selection was made based on NearIRDead cell staining and only alive cells were included in the analysis. Subsequently, a homogeneous population of single cells was selected according to morphological parameters. The percentage of CD8+ and CD4+ T cells in each tumor was calculated from the CD45+/CD8+ and CD45+/CD4+ double positive cell populations, respectively. The double positive cells CD45+/CD4+ were subsequently selected for Treg analysis using anti-Foxp3+ antibodies. Finally, MDSCs were identified by selecting the CD45+ cell population and analyzing their positivity to CD11b and Gr1 staining.

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MINI REVIEW
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Synthetic Gene Expression Circuits for Designing Precision Tools in Oncology

Angela Re *

Centre for Sustainable Future Technologies, Istituto Italiano di Tecnologia, Torino, Italy

Precision medicine in oncology needs to enhance its capabilities to match diagnostic and therapeutic technologies to individual patients. Synthetic biology streamlines the design and construction of functionalized devices through standardization and rational engineering of basic biological elements decoupled from their natural context. Remarkable improvements have opened the prospects for the availability of synthetic devices of enhanced mechanism clarity, robustness, sensitivity, as well as scalability and portability, which might bring new capabilities in precision cancer medicine implementations. In this review, we begin by presenting a brief overview of some of the major advances in the engineering of synthetic genetic circuits aimed to the control of gene expression and operating at the transcriptional, post-transcriptional/translational, and post-translational levels. We then focus on engineering synthetic circuits as an enabling methodology for the successful establishment of precision technologies in oncology. We describe significant advancements in our capabilities to tailor synthetic genetic circuits to specific applications in tumor diagnosis, tumor cell- and gene-based therapy, and drug delivery.

Keywords: synthetic circuit, biological engineering, synthetic biology, precision medicine, tumor diagnosis, tumor therapy, drug delivery, drug discovery

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***Correspondence:**
Angela Re
angela.re@iit.it

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INTRODUCTION

Synthetic biology builds on the transformative assertion that engineering approaches could be used to elucidate design principles of cellular systems and to implement synthetic digital and analog subsystems for a variety of end settings including health applications (Lienert et al., 2014). Since its beginning as a formalized engineering paradigm, which could be envisioned near the turn of the century when bacterial cells were programmed with basic genetic circuits (Gardner et al., 2000; Cameron et al., 2014), synthetic biology has provided a rigorous mechanistic foundation extremely helpful to quantitatively characterize the basic functions that are performed by the simple parts of a system and that collectively dictate the emergence of natural and human-defined phenotypes (Mukherji and van Oudenaarden, 2009; Elowitz and Lim, 2010). Nowadays, synthetic biology has greatly expanded in outlook, arising expectations, and stream of thought owing to the increasing intensive convergence of multifaceted engineering, life science, and biotechnology subfields.

The rapid progresses ensued from basic and applied synthetic biology research hold great promise in many contexts of substantial scientific and economic interest. The objective of this review is to reflect on the applications relevant to develop solutions to some of the challenges put forward by precision oncology. The precision paradigm that is being variously adopted by oncology

refers both to the chances for enhanced resolution and clarity in tumor identification as well as to the implementation of therapeutic interventions that could be set up on individual case basis (Jain, 2013; Kis et al., 2015). In this text, we provide an overview of synthetic genetic circuits engineering that apply to precision oncology and take advantage of the tight molecular control operating at multiple levels of gene expression (Vazquez-Anderson and Contreras, 2013; Fern and Schulman, 2017), through signal amplification, feedback, oscillatory, and logic capabilities (Wang et al., 2013; Lienert et al., 2014). Specifically, we show that engineered gene regulatory circuits are widening the assays available to report on tumor state and anti-tumor drug responses as well as to devise localized therapeutic options; for instance, increasingly advanced studies are being published on engineering cell classifiers (Morel et al., 2016; Mohammadi et al., 2017) and synthetic constructs for local payload delivery (Wagner et al., 2016). Furthermore, multiple gene-and cell-based therapy choices enhanced by synthetic biology applications are here described (Lim and June, 2017).

A great deal of efforts has been applied to investigate the rules of gene expression by precise measurements afforded by artificially constructed systems (Mukherji and van Oudenaarden, 2009). Much of the early contributions have focused on detailed and quantitative views of transcriptional regulation (Hockenberry and Jewett, 2012), and proceeded in tandem with experimental breakthroughs such as the use of combinatorial promoter libraries (Gertz et al., 2009). Nevertheless, substantial progress has also been achieved in ascertaining other regulatory mechanisms including post-transcriptional, translational, and post-translational modifications (Isaacs et al., 2004; Grilly et al., 2007). Almost all of these regulatory mechanisms are applicable to design gene regulatory platforms with controllable and predictable behaviors. Building on natural examples of regulatory circuits known to tune transcriptional and post-transcriptional activity (Cora et al., 2017), synthetic devices have demonstrated to modulate malignant phenotypes. Interesting examples here include synthetically engineered microRNAs targeting the MYC proto-oncogene (c-Myc) gene, which were shown to inhibit proliferation and induce apoptosis in bladder cancer cells (Fu et al., 2015), and the usage of aptamers to induce tumor cell death by destabilizing the apoptosis regulator bcl-2 (Soundararajan et al., 2008).

While the approaches to design the synthetic biological circuits that will be described could greatly vary, it is clear that abstraction, standardization (Galdzicki et al., 2014), and modularity (Endy, 2005) have been essential to formalize the design of such a broad range of gene expression systems and to handle biological complexity. Such principles lie behind many synthetic circuits to develop diagnostic and therapeutic tools, where basic parts such as promoters, gene coding sequences, terminators, and ribosome binding sites are assembled into modules such as toggle switches (Gardner et al., 2000; Niederholtmeyer et al., 2013) oscillators, and cascades (Davidsohn et al., 2015) to create predictable and continuously more sophisticated functionalities. The achievement of general and scalable systems (Weinberg et al., 2017) capable of sensing, reacting to, and controlling multiple component activities *in vivo* have required advanced programming paradigms to overcome barriers such as metabolic load (Weinberg et al., 2017), crosstalk (Huh et al., 2013; Kosuri et al., 2013; Trosset and Carbonell, 2013; Brewster et al., 2014), resource sharing (Cardinale et al., 2013; Segall-Shapiro et al., 2014), and gene expression noise (An and Chin, 2009) and thus to grant stability, robustness, and reliability of the engineered systems (Green et al., 2017).

The review is structured in two main sections. The former section summarizes engineering principles that are being applied to devise synthetic genetic circuits. Here, molecular tools exploiting transcriptional, post-transcriptional/translational, and post-translational control mechanisms of gene expression are discussed in separate subsections. The latter section describes specific areas of diagnostic and therapeutic technologies within the precision oncology enterprise where the potential of synthetic biology applications sits at the vanguard.

FROM GENE SWITCHES TO COMPUTING DEVICES

Biological engineering has enlarged the molecular tool set available to customize multicomponent constructs with increasingly varied and improved options for controlling gene expression. In particular, a great deal of design effort on synthetic gene switches has allowed to engineer cells with the capacity to sense, process, and switch gene expression state in response to intra- and extracellular signals. Engineering such sensing-actuating constructs involves linking a sensor part that detects the ligand to an actuator part that controls gene expression. The molecular design principles that have been used to customize synthetic gene switches differ according to the gene expression stage at which the switch is applied as well as on the distinctive properties that come with the choice of the switch constitutive parts (**Figure 1**).

Tools for Transcriptional Control

Circuits based on transcriptional control make up the largest number of synthetic circuits and share a common design, where an actuator part enabling positive or negative regulation of transcription is connected with a DNA-binding part that recognizes a promoter DNA sequence. Upon binding of a ligand, a sensor part triggers the activity of this complex through tethering or allosteric mechanisms (Ausländer and Fussenegger, 2013). While native transcription factors have come a long way in synthetic biology applications, it was not until the arrival of programmable transcription factors that it was possible to enhance the engineering capabilities of human-defined transcriptional switches. For example, Zinc-Finger (ZF)-containing factors (Khalil et al., 2012), Transcription Activator-Like Effectors (TALEs; Sanjana et al., 2012; Li et al., 2015), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based regulators (Bikard et al., 2013; Qi et al., 2013; Ferry et al., 2017) can be engineered to bind to specific DNA sequences of interest. Each class of TFs comes with advantages and disadvantages and is ideally suited to different applications (Jain, 2013). Major limitations

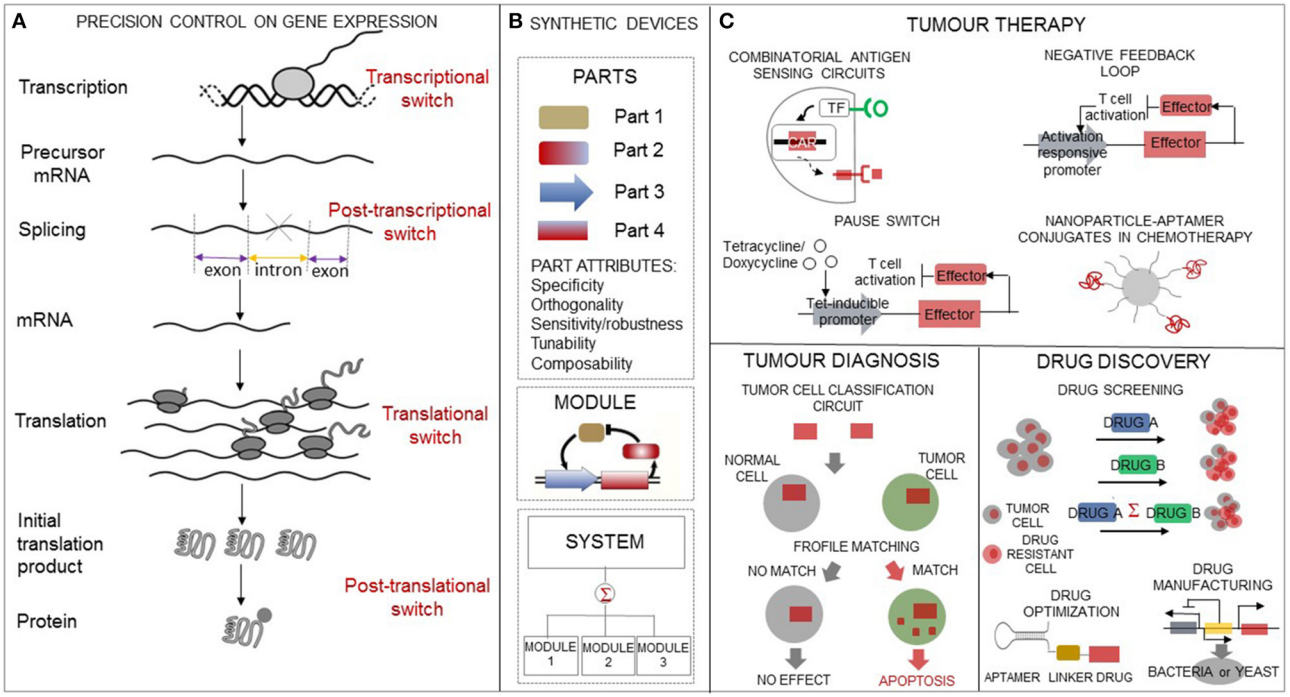

FIGURE 1 | Biological engineering enacts precision tools in oncology. **(A)** The synthetic biology toolbox contains a variety of regulatory switches which allow gene expression control at transcriptional, post-transcriptional, translational, and post-translational levels. **(B)** Abstraction hierarchy used for synthetic circuit design and construction. The hierarchy includes: *parts*, which are endowed with basic biological functions, *devices*, which are any combination of parts that perform a human-defined function, and *systems*, which are any combination of devices. **(C)** Overview of synthetic circuits' applications ranging from drug discovery to tumor diagnosis and to tumor therapy relevant to precision oncology interventions.

in the application of ZF-containing factors on synthetic circuits are their limited modularity and the lack of specificity of some ZF domains. TALEs are more straightforward to design than ZFs even though they pose challenges to cloning and delivery into host genomes. The CRISPR-based regulators are easier to construct than TALEs which, nonetheless, perform better for the construction of layered circuits (Lebar and Jerala, 2016). The plasmid pT181 antisense-RNA-mediated transcription attenuation platform is well established to control transcription through RNA–RNA interactions (Lucks et al., 2011).

Tools for Post-transcriptional Control

Due to its functional diversity, RNA is an advantageous substrate for information sensing, processing, and computation functions. Furthermore, the transient nature of RNA is appealing for applications where safety is a primary concern, since RNA-mediated circuits do not leave a long-term genetic footprint. RNA-based sensing-actuation switches are generally composed of highly folded sensor RNAs (aptamers) that, through conformational changes induced by the binding of small molecules or proteins, regulate the activity of RNA actuators that can operate *in cis* or *in trans*. Switches can sometimes rely on a transmitter part to transduce information between the sensor and actuator (Ogawa and Maeda, 2008). Aptamers have been engineered to respond predominantly to small molecules and nucleic acids (Werstuck and Green, 1998; Win et al., 2009; Shen et al., 2015) with extreme specificity whereas aptamers sensing proteins are far less intensely exploited (Culler et al., 2010).

Actuation can occur through diverse mechanisms including splicing, stability, translation, and mRNA localization. Owing to the known impact of ribonucleases (RNases) on RNA maturation and stability, aptamers have often been combined with RNA substrates for RNase activities (Vazquez-Anderson and Contreras, 2013; Comeau et al., 2016). Many RNA-based devices combine aptamers with catalytic actuators such as self-cleaving ribozymes to achieve flexible regulatory properties to fit application-specific performance requirements (Win and Smolke, 2007; Chen et al., 2010; Ketzer et al., 2012). Aptamers were also used with RNA interference substrates to control target mRNA silencing by regulating Drosha processing of pri-miRNAs (Beisel et al., 2011) or Dicer processing of small hairpin RNAs in response to endogenous signals (Saito et al., 2011). Furthermore, siRNAs and miRNAs have been shown to provide valuable options to implement Boolean logic frameworks (Rinaudo et al., 2007; Xie et al., 2011; Schreiber et al., 2016). A variety of switches have been developed to regulate translation of an open reading frame in response to the binding between the aptamer and small molecule (Stoltenburg et al., 2007; Wroblewska et al., 2015) or protein ligand (Hanson et al., 2003; Win and Smolke, 2007). Translation-control switches mainly affect translation initiation, such as the translational repression/activation switches consisting

of the ribosomal protein L7Ae and its box C/D kink-turn binding RNA motif (Saito et al., 2010, 2011). Furthermore, engineered systems can repress and/or activate translation by inducing conformational changes in nascent structured mRNA that modulate the access of the translational machinery to ribosome binding sites (Isaacs et al., 2004; Salis et al., 2009). Enhancement of protein synthesis has been recently achieved by the use of natural and synthetic antisense long non-coding RNAs (Yao et al., 2015) which were named SINEUPs due to the requisite of the inverted SINEB2 sequence to UP-regulate gene-specific translation (Zucchelli et al., 2015). Finally, engineering upstream Open Reading Frames (uORFs), whose regulatory potential is increasingly being appreciated (Re et al., 2016), is predictably an additional exploitable tool for protein manufacturing (Ferreira et al., 2013). Finally, RNA-based devices have been built to enhance gene regulatory activities through co-localization (Lee et al., 1999).

Artificial signal cascades can be constructed by combining multiple regulators, examples of which are inverter modules for synthetic translational switches (Endo et al., 2013). Programming Boolean operators for translational regulation has also been allowed by rationally designed variants of the RNA-IN-RNA-OUT antisense RNA-mediated translation system (Mutalik et al., 2012) as well as by the design of multiple orthogonal ribosome-mRNA pairs (Rackham and Chin, 2005), which were also implemented to synthesize orthogonal transcription-translation networks (An and Chin, 2009).

Tools for Post-translational Control

Synthetic switches have been designed that control protein activity by altering protein stability, which for instance is obtained by temporarily tagging proteins with a degradation signal, which guides the protein to the endogenous ubiquitin-proteasome system (Los et al., 2008; Collins et al., 2017). Efforts to engineer phosphorylation-mediated circuitry have been undertaken to rewire and construct MAP kinase circuits (Bashor et al., 2008; Wei et al., 2012; Ryu and Park, 2015). Additionally, the ability of inteins to form and cleave specific peptide bonds is extensively exploited to implement sensors of protein-protein interactions and small molecules, to realize synthetic circuits to deliver CRISPR-Cas9 system components (Truong et al., 2015) and to implement logic gates (Schaerli et al., 2014). Further efforts are ongoing to engineer and characterize synthetic compartmentalization approaches providing veritable solutions to implement modularity in synthetic devices (Chen and Silver, 2012).

SYNTHETIC CIRCUIT-BASED TOOLS FOR PRECISION MEDICINE IN ONCOLOGY

We outline synthetic biology applications which are expanding existing options in cancer diagnosis, cancer therapeutics, and for pharmaceutical compound screening (**Figure 1**).

Tumor Diagnosis

Precise cell state discrimination is essential for *in vivo* targeting of cancer cells. Medical diagnosis based on individual elements is unavoidably thwarted by lack of specificity and sensitivity. Therefore, diagnostic algorithms are being formalized using combinatorial Boolean logic to perform integrated detection and analysis of multiple signals in living cells (Rubens et al., 2016; Schreiber et al., 2016). Expression profiles are widely used to drive decision-making circuits such as the multi-input RNAi-based logic circuit identifying specific cancer cells (Xie et al., 2011). The cancer classifier circuit implemented in this study selectively triggers either a fluorescent reporter or apoptosis in HeLa cells. More precisely, this circuit integrates sensory information from six endogenous microRNAs to determine whether a cell matches a pathological reference pattern characteristic of the HeLa cervical cancer cell line and, if so, produces an apoptotic response. Early efforts to develop bio-based computing capabilities such as counting (Friedland et al., 2009) and memory storage (Siuti et al., 2013) lead to the notion that bacterial cells could become diagnostic indicators for recording exposure events (Cronin et al., 2012). In one of such studies, probiotic bacteria were transformed with a dual-stabilized, high-expression lacZ vector, and an integrated luxCDABE cassette endowing luminescent visualization in order to target, visualize, and diagnose liver metastasis (Danino et al., 2015). A recent study brought whole-cell biosensor closer to clinical requirements by configuring digital amplifying genetic switches, based on transistor-like three terminal devices (Bonnet et al., 2013), to actuate logic gates in bacterial chasses (Courbet et al., 2015). Here, digital amplifying switches are used in Boolean logic gates to perform complex signal processing tasks such as multiplexed detection of clinically relevant markers, signal digitization, and amplification along with storage of the medically informed outcome in a stable DNA register for *a posteriori* interrogation. Standardized devices for cancer diagnosis require a great deal of fine-tuning efforts to make combinatorial logic gates to perform as intended. Therefore, progressively advanced studies are being reported, opening interesting avenues to the automation of combinatorial circuit engineering (Ausländer et al., 2012; Nielsen et al., 2016; Weinberg et al., 2017). Even so, there are cumbersome problems that still need to be dealt with. Despite the breadth and depth described above, it is difficult to control the trade-off between specificity and sensitivity achieved by expression-based cell classifier designs, the changes in constructs performance dependent on genetic context, space and time as well as the possible toxicity induced by regulators overexpression. Balancing these problems must be addressed in order to allow synthetic gene constructs to become part of a personalized cancer therapy toolbox.

Tumor Therapy

Synthetic biology is primed to provide the conceptual framework and genetic tools necessary to enhance cell- (Fischbach et al., 2013) and gene- (Costales et al., 2017) based therapeutics.

Cell-Based Therapeutics

Immunotherapy has shown great promise for eradicating tumor in clinical trials. Much of the current success derives from therapies based on engineering T cell receptors (TCRs) and chimeric antigen receptors (Wilkie et al., 2012; Kloss et al.,

2013; Duong et al., 2015; CARs), that consist of a cancer antigen-specific single-chain variable fragment (scFv) fused to a T cell signaling domain that triggers activation and proliferation. Nowadays, synthetic sensors, switches, and circuits are primed to improve T cell therapy efficacy and meet safety concerns (e.g., discriminative capacity between tumors and vital organs and potential adverse side effects) by providing inducible control over the specificity, localization, duration, and extent of T cell activities.

Receptor systems

One of the most important challenges is represented by cell specificity. A powerful way to enhance on-target activity of therapeutic T cells is to engineer combinatorial receptor systems such as dual receptor AND-gate T cells (Roybal et al., 2016). In the antibody-coupled T cell receptor (ACTR) system, the scFv is replaced with the extracellular portion of CD16, a receptor that binds to the constant fragment of antibodies so that any relevant cancer-specific antibody can, in principle, be administered upon antigen binding (Kudo et al., 2014). Another major concern is the potential risk of unpredictable therapeutic effect. To enhance controllability, the recent GoCAR-T system incorporates a switch that activates CAR T cells when it is triggered not only by the target antigen expressed on the surface of the cancer cells but also by controlled administration of the drug rimiducid (Foster et al., 2017).

Control switches and circuits

T cell therapies could meet safety concerns if it were possible to eliminate quickly the engineered cells upon adverse side effects. Drug-inducible kill switches are an interesting development to achieve this goal. A recent example employs an inducible caspase 9 in conjunction with a CD20-specific CAR to test *in vivo* its potential to remove CAR-bearing T cells (Budde et al., 2013). Another study proposed to fuse caspase 9 to a modified FK-binding protein in order to allow conditional dimerization. This construct was proven to lead to cell death when exposed to a dimerizing small molecule (Di Stasi et al., 2011).

The design of negative feedback loops and inducible pause switches is proving a useful alternative to T cell elimination by modulating the immune response amplitude and timing. These circuits exploit the ability of bacterial virulence effector proteins to evade the immune response. The former type creates a negative feedback loop by expressing these proteins under the control of a T cell activation responsive promoter (Wei et al., 2012). The latter type of circuits pauses T cell activation by expressing bacterial virulence proteins under the control of a tetracycline inducible promoter. Indeed, adding the drug leads to the expression of the effector proteins, which in turn stop cell activation until the drug is removed (Wei et al., 2012).

Finally, a potent tool to regulate the therapy safety and efficacy is provided by growth switches (Chen et al., 2010). Here, a ribozyme drives self-cleavage of the cytokine transcript and leads to cytokine expression shut off; adding a proper drug prevents self-cleavage so that cytokines are expressed and lead to T cell proliferation.

Gene-Based Therapeutics

Gene circuit engineering has greatly improved our ability to programme genes involved in tumor origin and progress. For instance, some high-affinity RNA aptamers against PPAR-δ, a lipid-sensing nuclear receptor involved in cancer (Kwak et al., 2009), β-catenin (Lee et al., 2006), and nucleolin (Soundararajan et al., 2008), could lead to reduction of tumor-forming potential. Furthermore, a computational workflow, that selects RNA motif-small molecule binding interactions by library-vs.-library screening (2DCS) and then mines them against RNA folds in the transcriptome, allowed to identify a small molecule inhibitor of an oncogenic non-coding RNA (Velagapudi et al., 2017). SiRNAs can also specifically bind to target genes but their application can be limited by the absence of effective vehicles. For this purpose, several studies have proposed the use of aptamers in siRNA expressing constructs as vehicles (Tai and Gao, 2016).

Drug Delivery

Today nanobiotechnology provides extremely versatile options to address the localized delivery of genetically encoded tools such as virus-based vectors modified to carry engineered payloads (Ryan et al., 2004; Li et al., 2005), oncolytic viruses exploiting dual promoter logics (Nissim and Bar-Ziv, 2010), and nanoparticle-aptamer bioconjugates (Farokhzad et al., 2006). Recently, (Douglas et al., 2012) described a shape-switching device for targeted transport of signaling molecules. The robotic DNA device consists of a barrel provided with DNA aptamer-based locks that open in response to the binding of cell type-specific antigen keys. Vibrant developments greatly enhance and wide the range of applicable dynamic DNA and RNA-based nanoparticles (Afonin et al., 2013; Edwardson et al., 2016) besides opening newer avenue to conjugate inter-dependent nanoparticles (Halman et al., 2017). Polymer materials responsive to external signals (Stuart et al., 2010) such as nanogels conjugated to ligands recognized by cell specific receptors (Oishi et al., 2007), virus-mimetic nanogels (Lee et al., 2008), and hydrogels based on ligand-responsive DNA–protein interactions (Christen et al., 2011) demonstrate the essential progress in the area. In the future, nanorobots could be routed toward the tumor by exploiting the tumor-homing ability of self-propelled bacteria, similar to a recent study (Katuri et al., 2017). Biological vesicles derived from mammalian cells have also attracted much attention for *in vivo* delivery (Yoo et al., 2011). In particular, exosomes (Wang et al., 2016) have been engineered to deliver chemotherapeutics to tumor tissue in mouse models for cancer (Tian et al., 2014).

Drug Discovery

Synthetic biology is helping to address previously unfeasible challenges the field of drug discovery. Progress on design of synthetic genetic circuits (Carbonell et al., 2014; Trosset and Carbonell, 2015) has opened the possibility of their use not only for production of drugs (Breitling and Takano, 2015) but also for the development of platforms for identification and validation of drug targets (Firman et al., 2012; Kasap et al., 2014) as well as for phenotypic cell-based screening approaches (Duportet et al., 2014) such as the screening for anti-cancer drugs presented

in Gonzalez-Nicolini et al. (2004), that discriminates between proliferation competent and mitotically inert cells and eliminates preferentially neoplastic ones. With this purpose, (Gonzalez-Nicolini et al., 2004) engineered a transgenic CHO-K1-derived cell line to enable G1-specific growth arrest conditioned on the tetracycline responsive overexpression of the human cyclin-dependent kinase inhibitor p27. Another study applied a one-bead-two-compound (OB2C) cell-based screening approach for the discovery of synthetic molecules that can interact with cellular receptors as well as enhance or inhibit downstream cell signaling (Kumaresan et al., 2011). The primary innovation of this system is represented by the usage of beads provided with two chemical molecules on the surface and a chemical tag to probe cellular responses. When cells are incubated with the OB2C library, a cell adhesion ligand captures live cells on each bead in the library. The bound cells can interface with the tethered OB2C library compounds and then be probed for specific cellular signaling pathways such as leukemic cell death responses (Kumaresan et al., 2011). Largely because of similar progresses in conceptual design and technologies, synthetic biology is being employed as a powerful way to identify drug mechanisms of action and to accelerate the development of drug combination-based approaches (Chandrasekaran et al., 2016).

CONCLUSIONS

In this review, we focus on advances in biological engineering which stimulated the development of innovative approaches for precision intervention in oncology. The growing contribution of synthetic biology to drug discovery as well as the widening availability of synthetic circuits, which are already being used in different human compatible cell types and animal models for safe operation of gene- and cell-based therapies, demonstrate the potential of future approaches integrating systems and synthetic biology tools to precisely match therapies to individual cancer patients.

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The Mouse Hospital and Its Integration in Ultra-Precision Approaches to Cancer Care

John G. Clohessy[1,2]* *and Pier Paolo Pandolfi*[2]*

[1] Preclinical Murine Pharmacogenetics Facility and Mouse Hospital, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States, [2] Cancer Research Institute, Beth Israel Deaconess Cancer Center, Department of Medicine and Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

Precision medicine holds real promise for the treatment of cancer. Adapting therapeutic strategies so patients receive individualized treatment protocols, will transform how diseases like cancer are managed. Already, molecular profiling technologies have provided unprecedented capacity to characterize tumors, yet the ability to translate this to actionable outcome in the clinic is limited. To enable real time translation of personalized therapeutic approaches to patient care in a co-clinical manner will require the adoption and integration of approaches that facilitate modeling of patient disease. The Mouse Hospital represents an approach that is ideally suited to pre- and co-clinical evaluation of novel therapeutic strategies for clinical care. Patient derived xenograft (PDX) technologies and *in situ* tumor modeling approaches using genetically engineered mouse models (GEMMs) already have a proven capacity to mimic human tumor responses, and their application can deliver invaluable insights into appropriate clinical approaches for individual patients by mirroring human clinical trials using a Co-Clinical Trial project and Mouse Hospital infrastructure. Additionally, the integration of the Mouse Hospital with other emerging technologies for the application of precision medicines, including organoid technologies, provides a platform that enables medical centers to truly reap the benefits that precision medicine has to offer.

Keywords: mouse models, PDX, precision medicine, cancer, Mouse Hospital, Co-Clinical Trial

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Lawrence Schook, University of Illinois at Urbana-Champaign, United States

***Correspondence:**
John G. Clohessy
jclohess@bidmc.harvard.edu
Pier Paolo Pandolfi
ppandolf@bidmc.harvard.edu

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Precision medicine has long been lauded to deliver the cure and eradication of diseases such as cancer, tailoring treatments to the specific genetics and needs of the patient. Indeed, the technological advances that we have seen over the 20–30 years have allowed us to profile and characterize patients and tumors to an unprecedented level, enabling a detailed mapping of genomic alterations and characterization of mutations observed in disease. Yet, the translation of these findings and approaches to the care and treatment of individual patients falls far behind the trailblazing advances in the technology that individualizes tumors. Much of this lag in translation to the clinic, lies in the historical approaches and methods in place for the testing and clinical evaluation of agents to be brought to the clinic, with a lack of infrastructure to facilitate translational studies in academic medical centers.

Our lab has pioneered the development and implementation of The Mouse Hospital and Co-Clinical Trial Project (1–4). This concept offers a mechanism by which tailoring of treatments and design of patient specific therapies can be rapidly evaluated. The Mouse Hospital encapsulates an infrastructure by which mouse trials can be carried out in a manner that mimics human trials

and treatments. In this setting, resources including imaging, treatment and pathology mirror human resources, and are integrated with standardized operating procedures and ongoing training of technical staff to ensure best practice and provide a recognized "standard of care" in mice that mimics human treatments. This in turn enables Co-Clinical Trials to be carried out in mice, whereby concurrent human/mouse trials mimic and inform one another. However, such an approach requires a number of important considerations from a practical perspective (3), and its integration within the context of a clinical trial and translational framework to benefit patients requires careful consideration. Indeed, the variety of models and their application offer a number of different and unique approaches that can be adopted and tailored to patient needs, and should be considered in the context of other precision medicine based technologies that offer the potential to identify unique therapeutic protocols for patient treatments. Here we outline key elements of the Mouse Hospital and the Co-Clinical Trial approach that can meet the needs of precision medicine, and discuss the challenges facing these approaches that need to be met to facilitate routine incorporation and utilization to deliver superior cancer patient care.

MODELING PATIENTS IN MICE

For the purposes of modeling human cancer in mice, there are currently two predominant approaches utilized. One represents the growth and expansion of tumor tissue in immunocompromised mice in a patient derived xenograft (PDX) or avatar setting, while the other represents genetically engineered mouse models (GEMMs), whereby the mouse genome is engineered to harbor key genetic alterations to drive tumor development *in situ* for the purposes of following tumor initiation and progression (5, 6). PDX tumor models have the advantage of studying human tumors themselves, enabling the expansion and evaluation of multiple single agent and combination therapies, however their immune compromised state fails to fully recapitulate the tumor microenvironment within which tumors exist. Although GEMMs may not always fully recapitulate the full heterogeneity and complex genetics of human patients, they do have the advantage of their *in situ* localization, and enable study of evolution and immune related function on tumor growth, progression and response to therapy. This is of particular importance in the context of immune-therapies, which represent a rapidly growing area for therapeutic intervention in many cancer types, and where novel immune targeting therapies require pre-clinical evaluation (7). Indeed, although both PDX and GEMM models have provided important tools for the study of human cancer, there are key challenges that still need to be met in order to provide a more robust and useful platform for integration with clinical studies.

While PDX models offer the opportunity to uniquely match individual patients with mouse avatars for evaluation of drug response to their unique tumor, not all patient tumors grow and progress in a xenograft setting (8–10). In addition, orthotopic vs. subcutaneous tumor implantation for development and propagation remains an issue of discussion (8, 10). Much of the work to date has focused on subcutaneous propagation of PDX tumors, providing easy access to follow tumor growth and monitor response to treatment, and while there is evidence that orthotopic propagation of PDX tumors may facilitate some tumor types, this frequently requires much greater technical expertise, and does not always lend easily to enrollment of tumors at similar stages, and the longitudinal monitoring of individual tumor types. More recently, evidence highlighting the limitations of PDX models to faithfully model human tumors has demonstrated that propagation of human tumors in mice can result in a distinct evolution of these tumors (11). Indeed, while this study noted that the degree of genetic instability between human tumors and PDX models shares similarities, the distinct copy number alterations (CNAs) that occur in the evolution of human vs. PDX tumors highlights how the murine environment promotes clonal selection distinct from that occurring in patients (11). This may have important implications for the reliability of PDX tumors as avatars for human disease and their use in co-clinical studies, particularly as arm-level CNAs can be associated with drug response, and clonal selection resulting from PDX propagation can impact CNAs present, and in turn influence therapeutic outcomes (11).

GEMM models have their own particular challenges and are somewhat limited by the number of genetic alterations and time required for development of tumors *in vivo*. This frequently prevents GEMM models from acting as individual patient avatars, but they approximate patients based on key genetic drivers or modifiers for a particular cancer type. However, the emerging role of immune cell types in cancer has highlighted the need for models to study and understand the relationship in cancer (7). Particularly in the context of therapy where agents targeting immune cells are emerging as key elements for cancer therapy, and increasing relevance of cancer vaccines in maintaining remission and preventing recurrent disease is gaining momentum (12–14). Indeed, GEMM models are also now highlighting how the genetics of the tumor can influence the immune landscape of tumors, and in doing so influence the tumor biology (15). However, adaption and refinement of GEMMs is required to better facilitate pre- and co-clinical trials in the context of the mouse hospital so as to provide a more off-the-shelf approach for their utilization and application to real-time patient trial integration. Abilities to more easily modify genomes utilizing CRISPR genome editing approaches are facilitating this transition, and new opportunities for the development of models and their application are emerging (16–20).

Thus, PDX and GEMMs models complement each other in what they have to offer the cancer patient. A combination of efforts that take into account the patients own tumor, with its heterogeneity and complex genetics, in addition to a more simplified and focused model that looks to the main genetic drivers to account for generalizations amongst tumor types, and that take advantage of both immune-compromised and -competent settings.

INTEGRATING MOUSE STUDIES WITH CLINICAL CARE

While efforts aimed at improving models to provide enhancement in their application to uncovering novel therapeutic approaches for cancer is ongoing, how these models inform patient care, and how they are integrated into the precision medicine framework is also of relevance (**Figure 1**). This requires integration of mouse modeling approaches with existing technologies that have been built to support patient care in the context of precision medicine. Of particular relevance are areas of cellular profiling related to DNA and RNA sequencing, proteomic, and metabolic analyses, as well as culture of primary tissue explants from cancer patients.

In the context of molecular profiling, advances in computational approaches to defining individual patient drug resistance or sensitivity have greatly improved. Large publicly available datasets that include The Cancer Genome Atlas (TCGA), Cancer Target Discovery and Development (CTD^2) database (21, 22), and the Cancer Cell Line Encyclopedia (CCLE) (23) have already demonstrated both the ability of molecular profiling to identify and hone in on key networks and pathways that provide insights on heterogeneity of tumors and facilitate identification of tumor sub-types, as well as highlighting how a central repository for datasets can facilitate analysis. Enabling data to be stored and input in a central repository can greatly facilitate co-clinical efforts involving multi-center co-clinical trials. Such a resource can extend beyond data sharing and analysis to also include shared protocols, relevant metadata, tools, and greatly facilitate research through web accessibility. Indeed, relevant DNA or transcriptome profiling for patients and models can enable comparison with big-data repositories to identify similarities with tumors already demonstrated to be sensitive or resistant to known chemotherapies or targeted agents (24). Such approaches facilitate the identification of focused therapeutic options for patients, and enable identification of targeted agents appropriate for defined genetic cancer types. In adapting such approaches, the evaluation and divergence of PDX models in particular from the original primary tumor should be followed, comparing CNA and transcriptomic profiles to account for clonal selection through PDX propagation. Indeed, it is possible that computational approaches can "correct" for responses in such situations, providing a statistical framework to facilitate translation of PDX response and outcome in co-clinical studies, to account for such divergence.

In general, while the long latency to generate and propagate PDX models for co-clinical studies provides a challenge for real-time application in this setting, organoid technologies are emerging as an efficient method by which to rapidly grow and expand primary tumors in culture. Current efforts to characterize these cultures as tumor models has highlighted their potential in study and evaluation of their representative patients (25–28). The ability to grow these primary tumors *in vitro*, enables a more high-throughput screening of individual patient tumors for sensitivity to drugs already approved for clinical use or under clinical evaluation. In addition, human organoid cultures can be utilized for the development of PDX tumors *in vivo*. and organoids derived from mouse primary tissues and cell types can provide useful models for human cancer (29–31). Thus, organoid approaches can facilitate with evaluation of tumor sensitivity to therapeutic agents, and enable the testing of

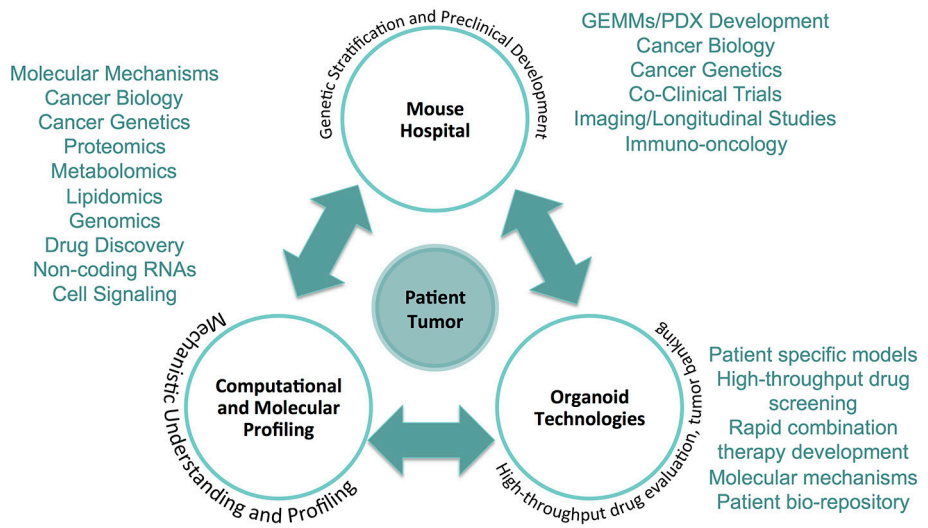

FIGURE 1 | The precision medicine framework integrating The Mouse Hospital. The Mouse Hospital ideally is integrated in an ultra-precision medicine framework whereby data collected from computational and molecular profiling of patient tumors, in addition to screening and characterization of primary cancer organoid cultures, enable prioritization of novel therapeutic strategies for *in vivo* pre- and co-clinical evaluation. The integration of each of these approaches provides a comprehensive platform that can deliver actionable therapeutic strategies for individual patients that goes above and beyond what is currently available based on targeted genomic sequencing.

multiple combinations of therapeutic agents to identify potential therapeutic strategies for the treatment of individual cancers.

Combining these approaches as first line co-clinical efforts aids in optimally integrating mouse model approaches for precision medicine. Indeed, such *in silico* and *in vitro* analysis enables a well-defined prioritization of therapeutic strategies that can be evaluated and validated *in vivo*. This streamlines the use and application of mouse modeling approaches for translation of novel therapeutic strategies to the clinic, and enhances the effectiveness of *in vivo* translation. Such a pipeline represents an attractive model for the execution of precision medicine for cancer patients, going beyond a simple genetic or transcriptome profiling approach to stratify patients for therapy, to providing an ultra-precision platform that tailors treatments to provide the most optimal therapeutic strategy.

How co-clinical and clinical efforts are integrated and inform one another is also of relevance. The use of patient material for mouse related studies in the context of pre- and co-clinical requires approvals from both Institutional Review Boards (IRB) and Institutional Animal Care and Use Committees (IACUC). Additionally, challenges surrounding patient privacy and how data generated and analyzed are stored needs to be carefully considered, and protocols implemented need to adhere to appropriate HIPPA guidelines if such studies are to directly impact patient care. This requires that institutional infrastructures be in place to ensure that data are properly protected and patient identification only accessible by appropriate clinical staff. Similarly, while more general studies carried out using GEMM models or de-identified PDX models within a co-clinical setting to evaluate response to novel therapeutic agents or combinations thereof, it is crucial that therapeutic response in these models be carefully correlated with relevant response in human patients as outlined below. This frequently requires that individual models are carefully optimized to ensure standardized application of the model to anticipate therapeutic outcome.

ULTRA-PRECISION MOUSE MODELS FOR CANCER CARE

Although efforts to utilize mouse models in such an integrated ultra-precision platform is an attractive approach to maximize efficacy of data generated from *in vivo* studies, and provide effective clinical approaches to treat cancer patients, it is essential that strict procedures and protocols are in place to ensure reproducibility and reliability across the platform (3, 32). Currently there are no clear guidelines for how mouse models should be integrated into translational studies that directly impact patient therapy, and several studies highlighting issues concerning reproducibility across academic research demonstrate the need for systems that ensure the reliability of such data. Thus, it is essential that standard operating procedures (SOPs) are generated and in place to provide appropriate quality systems founded on good laboratory practices (GLP) that include detailed protocols, reporting and archiving to ensure all relevant data are recorded for reference and repeatability. It is also important that quality assurance units be included as part of the systems in place to ensure conformation with GLP. Such a GLP approach ensures uniformity and consistency in the performance of relevant studies, and facilitates evaluation of systems in place by regulatory authorities. Indeed, the Organization for Economic Co-operation and Development (OECD) already provides guidelines for testing and evaluation of chemicals that can be readily adapted for co-clinical use (33, 34). In addition, it is essential that appropriate education and training are provided to those carrying out such studies, and that records and data are appropriately maintained and archived. Ultimately, pre- and co-clinical studies involving mice will be carried out in a Clinical Laboratory Improvement Amendments (CLIA) approved environment to facilitate the approval and translation of studies from the bench to the bedside.

It is also important that such approaches be considered and evaluated by internal review boards (IRB), who oversee and approve ongoing clinical trial protocols within the academic medical setting. The ability to inform patient care in real-time, through precision medicine approaches offers unique opportunities for cancer patients, and translating novel therapeutic strategies to the clinical for individual patients based on a cohort of pre- and co-clinical studies requires careful evaluation to ensure patients are protected and offered treatments that truly represent best-option strategies for their specific cancer. In translating these results, the ability to match or predict how response in mouse models equates to a response in human patients is of great importance. The use and application of RECIST (Response Evaluation Criteria In Solid Tumors) and irRECIST (immune-related RECIST) criteria in patients has become an essential set of rules that define patient response to treatment. Equating responses in GEMM and PDX models to appropriate RECIST responses in patients can require optimization and may be developed through iterative processes, but can dramatically improve the clinical relevance of co-clinical studies. This requires that pilot studies be carried out to properly establish an appropriate treatment regime corresponding to patient treatments, which almost invariably includes upfront standard of care therapies. It is therefore important not to jumpstart the process by solely evaluating experimental therapies, but always evaluating and correlating mouse model response to standard of care as appropriate for the relevant clinical trial. This in turn can set clear criteria for evaluating response and subsequently be utilized to support the use of such models in clinical trial protocols, again however, clear criteria and GLP approaches are necessary to ensure reproducibility and reliability as outlined above.

As part of such an approach, it is imperative that drugs and therapies used in the pre- and co-clinical setting mimic as closely as possible those that will ultimately be administered to patients. However, it may not always be the case that such agents can be easily assessed, particularly in the case of GEMMs. Many human specific therapies, including biologics or small molecule inhibitors, demonstrate limited cross-reactivity or specificity for mouse targets, and thus lack of efficacy in such models requires that mouse specific reagents be generated (35). However, the enrollment

of mouse models for the purposes for testing of novel therapeutic approaches can greatly facilitate evaluation of both targeted agents, and evaluation of combinatorial therapies. This can greatly aid rapid stratification and testing of multiple therapeutic options, in turn tailoring therapies for patients. Similarly, it is important to consider dosing strategies for corresponding mouse and human trials, and integration of mouse models can provide insights on differential dosage as well as metronomic therapy approaches for clinical application. Indeed, a number of studies have demonstrated the usefulness of mouse models in optimizing dosing strategies for patients to deliver more effective responses to standard cancer therapies (36, 37).

FUTURE DIRECTIONS

As outlined above, despite extensive advances in technologies that support cancer patients and the ability to characterize their unique cancer, there is a critical need to go beyond utilization of this resource as simply a diagnostic or prognostic tool. While currently such data provides an actionable therapeutic option in limited cases, often reserved for specific targetable mutations, all too frequently much of the information gleaned provides little therapeutic value (38). Thus, the integration of such data with computational and molecular databases, combined with *in vitro* screening and characterization of primary disease utilizing organoid technologies, can be readily translated to the clinic through *in vivo* validation using mouse models (2, 4, 39). It is also of paramount importance to include global genomic and transcriptomic analysis toward more accurate predictions, as well as for the identification of novel mechanisms of resistance as recent studies indicate (40). Development of such a platform to integrate patient and mouse hospitals through co-clinical studies provides a clear pipeline for delivery of ultra-precision solutions for individual cancer patients (41). Such an approach requires careful organization and set-up to ensure such models provide accurate insights for development of patient care strategies and represent a key component of precision medicine centers of the future.

AUTHOR CONTRIBUTIONS

JGC and PPP conceived, researched, and co-wrote the manuscript.

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