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TITLE: Polyploid Giant Cancer Cells actuate prostate cancer tumor resistance and lethal phenotype

PRINCIPAL INVESTIGATOR: Sarah Amend

CONTRACTING ORGANIZATION: Johns Hopkins University

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6. AUTHOR(S)
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14. ABSTRACT

Lethal prostate cancer is incurable because the population of cancer cells within a tumor are resistant to all known compounds, including standard-of-care therapy. Resistance to a therapy may be solely cell intrinsic, therefore present in a treatment-naïve setting, as well as be induced through external selective pressure via therapeutic treatment. How and when drug resistance arises has profound implications to understand tumorigenesis as well as to guide treatment and management of disease. We and others have have demonstrated that the appearance of a subset of morphologically distinct cancer cells with high genomic content, polyploid giant cancer cells (PGCCs), is associated with therapeutic interventions. Based on our preliminary results, we hypothesize that PGCCs are central mediators of tumor resistance and prostate cancer lethality. To test this hypothesis, we will: 1) determine how and when PGCCs are formed during cancer progression and 2) demonstrate that quiescence underlies a common mechanism of stress resistance in PGCCs. The results of the above studies will fundamentally change our understanding of how and when therapeutic resistance arises during prostate tumorigenesis and its treatment course, specifically delineating the role of polyploid giant cancer cells as mediators of therapeutic resistance in prostate tumors. In the current term, we have begun to understand the mechanisms of PGCC formation resulting in a single nucleus or multiple nuclei. We have shown that PGCCs arise under multiple tumor microenvironmental stressors, including hypoxia and altered pH. We found that PGCCs under therapy exist in a G0 quiescent state. Finally, we have demonstrated that PGCCs have stem like properties, both in terms of function and molecular markers. This work has resulted in multiple peer-reviewed publications in the last year.

15. SUBJECT TERMS
Therapy resistance, polyploidy, polyaneuploid cancer cells, polyploid giant cancer cells, quiescence

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1. Introduction

Lethal prostate cancer is incurable because the population of cancer cells within a tumor are resistant to all known compounds, including standard-of-care therapy. Resistance to a therapy may be solely cell intrinsic, therefore present in a treatment-naïve setting, as well as be induced through external selective pressure via therapeutic treatment. How and when drug resistance arises has profound implications to understand tumorigenesis as well as to guide treatment and management of disease. We and others have have demonstrated that the appearance of a subset of morphologically distinct cancer cells with high genomic content, polyploid giant cancer cells (PGCCs), is associated with therapeutic interventions, including taxane-based chemotherapy in castrate resistant prostate cancer. The presence of PGCCs has been recognized for more than a century, but significance of these cells in tumor biology has been largely unexplored. Based on our preliminary results, we hypothesize that PGCCs mediate resistance and survive by existing in a dormant quiescent state and, upon a period of recovery, give rise to a resistant a resistant population of non-polyploid cells. Therefore, we hypothesize that PGCCs are central mediators of tumor resistance and prostate cancer lethality. To test this hypothesis, we will: 1) determine how and when PGCCs are formed during cancer progression and 2) demonstrate that quiescence underlies a common mechanism of stress resistance in PGCCs. The results of the above studies will fundamentally change our understanding of how and when therapeutic resistance arises during prostate tumorigenesis and its treatment course, specifically delineating the role of polyploid giant cancer cells as mediators of therapeutic resistance in prostate tumors.

2. Keywords

Therapy resistance, polyploidy, polyaneuploid cancer cells, polyploid giant cancer cells, quiescence

3. Accomplishments

What were the major goals of the project?

Specific Aim 1. Determine how and when PGCCs are formed during prostate cancer progression

Major Task 1: Measure PGCCs in prostate cancer tissues in murine models.

Major Task 2: Measure PGCCs in patient TMAs

- Milestone #1: generate a novel prostate cancer progression TMA
  Goal: month 20
  This Milestone has been delayed due to COVID-19-related delays and delays with human subjects research approval. New goal date: month 30.

Major Task 3: Determine the tumor microenvironment conditions to induce formation of PGCCs.

Major Task 4: Determine mechanism of PGCC formation

- Milestone #2: Submit manuscript on the role PGCCs in prostate cancer progression, including as a prognostic factor of disease progression/ response to therapy and how PGCCs are initially formed.
  Goal: Months 30-36
  This Milestone is on-track.

Specific Aim 2: Demonstrate that quiescence underlies a common mechanism of stress resistance in PGCCs

Major Task 1: Generate cell cycle reporter prostate cancer cell lines to be used in subsequent experiments

- Milestone #3: Develop and validate stable PC3 cell lines for prostate cancer research community that can be used to monitor quiescence entry/exit in vitro and in vivo.
  Goal: Month 20
  This Milestone has been delayed approximately 6-8 months due to COVID-19-related delays, hiring delays, and technical issues.
  New goal date: Month 27

Major Task 3: Examine quiescence markers in PGCCs under other treatment conditions that lead to resistance.

Major Task 4: Evaluate stem-cell properties of PGCCs.

- Milestone #4: Submit manuscript on entry and exit of quiescence and resistance in polyploid giant cancer cells in prostate cancer
  Goal: Months 24-36
  This Milestone is on-track.

What was accomplished under these goals?

Specific Aim 1. Determine how and when PGCCs are formed during prostate cancer progression

Major Task 1: Measure PGCCs in prostate cancer tissues in murine models.

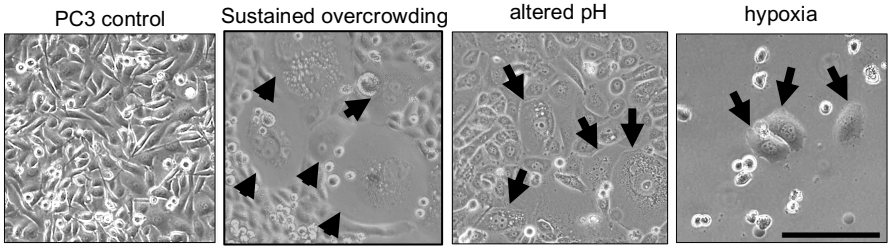
Due to unexpected and unavoidable COVID-19-related restrictions, mouse colonies were substantially reduced to only include breeding pairs to maintain the GEMM lines. After these restrictions were lifted, it took several months to expand the colony to the point that aging of the GEMM models with the appropriate N could be accomplished. This expansion/aging is currently underway.

Major Task 2: Measure PGCCs in patient TMAs

Due to COVID-19-related delays and delays specifically due to misunderstanding of regulation, the determination of PACC index has not been accomplished. In the interim, however, antibody staining has been validated, workflow of samples has been optimized, and workflow of imaging analysis by the pathology fellow has been accomplished. As such, we are poised to begin this work immediately upon approval.

Major Task 3: Determine the tumor microenvironment conditions to induce formation of PGCCs.

We have found that PGCCs can be formed in response to multiple different external stressors commonly found in the tumor microenvironment including: low oxygen (induced via altered O2 concentrations and by chemical mimic of hypoxia CoCl2), altered pH (induced via altered CO2 concentrations), and severe sustained overcrowding.



PGCCs are enriched following exposure to TME stressors. PC3 cells were cultured under conditions of sustained severe overcrowding (overconfluent for 20 days), altered pH (14d) or in hypoxia (14d). PACCs indicated by arrows (not exclusive; scale = 200 um).

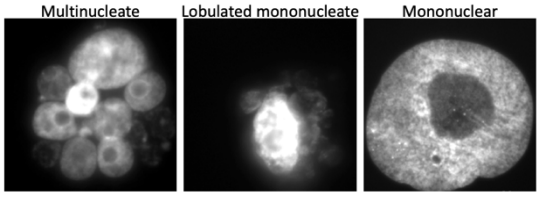
Major Task 4: Determine mechanism of PGCC formation.

PGCCs can be formed via a number of evolutionarily- and developmentally-conserved mechanisms (which we published in: doi: 10.1016/j.semcancer.2020.11.016). These include cell-cell fusion, endocycling, mitotic slippage, and late or early endomitosis.

To investigate the frequency of cell-cell fusion vs cell-autonomous mechanisms, we utilized cell lines engineered to express GFP or mCherry. After PGCC induction with chemotherapy or with hypoxia, we did not find evidence of cell-cell fusion by imaging or by flow cytometry. This negative data suggests that fusion is a very rare event for PGCC formation in our models.

To confirm these data, we performed time-lapse imaging of PGCC formation. These experiments are difficult to perform as the likelihood of capturing the moment of PGCC formation and the appropriate field of view over several days is non-trivial. However, in initial experiments, we have only observed failed cytokinesis, i.e., late endomitosis. Further work is needed to define these mechanisms specifically.

In other imaging studies, we acquired incidental data that PGCCs can be categorized as three types: single uniform nucleus, single lobulated nucleus, and true multinucleation. Work is currently underway to determine the relative ratios of these PGCC types and to track the fate of each type to determine if one has preferential survival under stress and/or depolyploidization potential.



PGCCs were stained with DAPI and imaged using confocal microscopy to attain 3D resolution. Three general nuclear forms were observed: multinucleated, a single lobulated nucleus, and a single uniform nucleus.

Specific Aim 2: Demonstrate that quiescence underlies a common mechanism of stress resistance in PGCCs

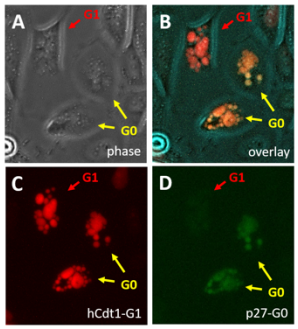
Major Task 1: Generate cell cycle reporter prostate cancer cell lines to be used in subsequent experiments

Initial work was done with the FUCCI++ reporters in prostate cancer cell lines. Following a set of initial experiments (the results of which are included below), these cell lines tested positive for mycoplasma during a routine check. All cells were discarded.

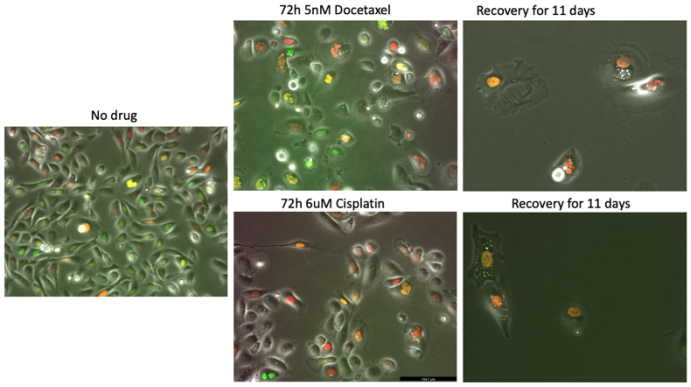
Cells are now being generated using the FUCCI++ reporters described in the proposal as well as a recently-published next-generation FUCCI reporter. Because hiring restraints slowed production of these cell lines, we are now taking advantage of a core facility to generate these cell lines in a timelier manner.

Major Task 3: Examine quiescence markers in PGCCs under other treatment conditions that lead to resistance.

In our initial experiments exploring FUCCI status of cells under treatment, we found that a subset of PGCCs were quiescent in G0 immediately after treatment with docetaxel or cisplatin. PGCCs were isolated and allowed to recover in chemo-free media for 11 days. The majority of PGCCs were double-positive (hCdt1 and p27), indicating that they were in G0.



PC3-G0-fucci cells treated with 6uM cisplatin for 72 hours. PGCCs show heterogeneity of hCdt1 and p27 fluorescence, resulting in production of both G1 and G0 PACCs under the same conditions.



PC3-G0-fucci cells treated with 5 nM docetaxel (top) or 6uM cisplatin (bottom) for 72 hours. PGCCs were isolated and allowed to recover for 11 days. Immediately following treatment, PGCC had a high heterogenetity of FUCCI signal. After 11 days in treatment, the majority of surviving PGCCs were double-positive, indicating that they are in the G0 cell cycle phase.

Major Task 4: Evaluate stem-cell properties of PGCCs.

PCa cell lines PC3, LNCaP, or DU145 cells with cisplatin for 72 hours (data for PC3). Visual inspection and flow cytometry revealed the emergence of PACCs. We isolated the PACCs via a novel filtration protocol (confirmed PGCC isolation by flow in the figure), removed the chemotherapeutic stress, and monitored population recovery. After a period of stress release, the recurrent population reverted back to the parental cell morphology and ploidy, and PACCs once again represented <5% of the total cell population. These data indicate that PGCCs generated by multiple stressors have the capacity to seed a recurrent population.

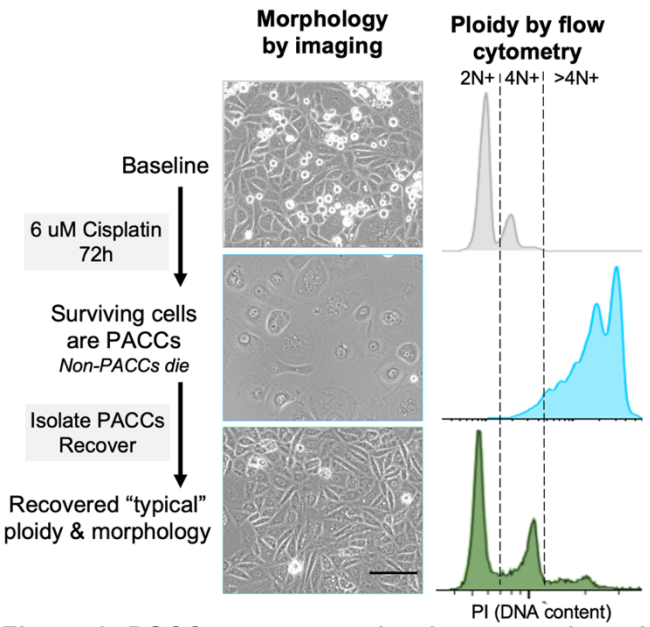
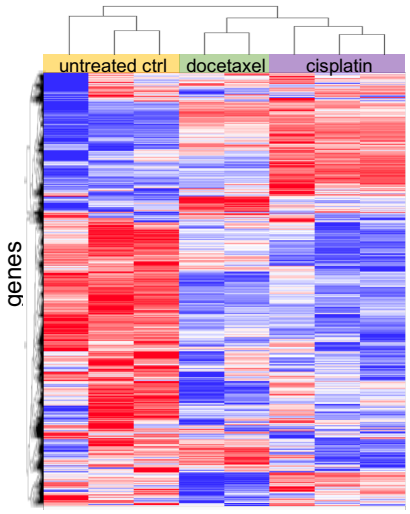
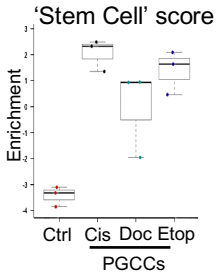


Figure 6. PGCCs emerge under therapy and seed recurrence. PC3 cells were cultured in 6 uM (IC50) Cisplatin for 72 h. Cell morphology was assessed by imaging (scale 100 um). Ploidy was assessed by flow cytometry cell cycle analysis (DNA stain PI). PGCCs were isolated and allowed to recover. The recurrent population had typical morphology and ploidy distribution.



PGCCs induced by different classes of chemotherapy share a transcriptional signature. PC3 cells were cultured with 5 nM docetaxel or 12 uM cisplatin for 72h. PGCCs were isolated, and RNA was isolated from control untreated PC3 and PGCCs and run on NanoString (PanCancer Progression Panel). Unsupervised hierarchical clustering of normalized and z-score transformed (clustered by gene using Euclidean distance) shows that the PACCs induced by docetaxel (green) or cisplatin (purple) segregate independently from control (yellow). Heatmap: columns=samples; rows=genes.



PGCCs are enriched for stem-cell markers. (A) PACCs isolated from PC3 cells treated with cisplatin, docetaxel, or etoposide were scored for a stem-cell signature (NanoString).

To begin to identify shared genes, proteins, and pathways involved in the PACC state, we used the NanoString PanCancer Progression panel to profile PACCs isolated from PC3 cells treated PC3 cells with IC50 concentrations of docetaxel, cisplatin, and etoposide for 72 hours compared to control. Strikingly, while each therapy had uniquely-differentially expressed genes as expected, many genes were upregulated in all PACC

populations, regardless of therapy class. To specifically assess the stem-like properties of the PGCCs, we performed nCounter pathways analysis and found that all PGCCs are enriched for a "Stem Cell" score.

One challenge for depolyploidization of polyploid cells is centrosome clustering to avoid mitotic catastrophe. We found that KIFc, the kinesin essential for centrosome clustering has increased expression in PGCCs. Using publically-availabile databases, we found that KIFc1 expression is asscoiated with poor prognosis in prostate cancer (doi: 10.1007/s12032-021-01494-x).

Lastly, we have performed limiting dilution assays of PGCCs formed in response to docetaxel and cisplatin. Though we have not observed the depolyploidization event, we have successfully isolated five populations that arise from a limiting dilution colony of PGCCs. Ongoing work is being done to confirm the origin of such colonies is a PGCC (vs a contaminating non-polyploid cell) and to observe the initial depolyploidization event.

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?

Results were presented to the scientific community in the form of manuscripts and presentations (listed below). Results were presented to non-scientific communities of interest via an interactive lecture as part of "Urology Speaks," a lecture series organized by the Chairman of The Brady Urological Institute, reaching more than 100 participants.

What do you plan to do during the next reporting period to accomplish the goals?

Over the next reporting period, we aim to 1) attain appropriate approval to move forward with TMA construction and analysis, 2) continue to expand the animal colonies to initiate GEMM modeling, and 3) continue the described *in vitro* and *in vivo* studies as outlined in the SOW. With all positions once again filled, we anticipate the proposed research to advance as initially projected.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
This work has contributed to the understanding of therapy resistance in cancer – in prostate cancer specifically as well as other solid tumors. This shift in paradigm is highlighted in several peer-reviewed published manuscripts from the last funding year (doi: 10.1073/pnas.2020838118; doi: 10.1016/j.semcancer.2020.11.016; doi: 10.1111/eva.12929)

What was the impact on other disciplines?

This work has informed the understanding of evolution of resistant cancer cells, directly impacting and informing research in evolutionary ecology and geochemistry (study of speciation through the geo-record). This is facilitated through weekly working group meetings to apply the PGCC research to these other fields, and this collaboration is proven by the publications outlined above.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change
No major changes in approach have been made or are planned.
Minor changes in approach:

1. New isolation strategy for PGCCs: Previously, PGCCs were isolated by flow cytometry, leading to bottlenecks in productivity related to FACS machine maintenance, time with machine, and greater cell numbers needed for high viability. While we still use FACS to confirm all results, we have also

developed a filtration protocol to distinguish PGCCs on the basis of size and deformability. We have confirmed that this approach results in a pure PGCC population.

2. The G0-FUCCI cell line was found to be mycoplasma positive during routine mycoplasma testing and cell line authentication. As such, we need to re-engineer the FUCCI lines. In 2020, a new FUCCI reporter was published (doi.org/10.1152/physiolgenomics.00065.2020) that is superior to the constructs we described in Specific Aim 2. We are in the process of generating cell lines with both constructs that will enable us to validate data with two independent G0 FUCCI reporters.

Actual or anticipated problems or delays and actions or plans to resolve them

We have encountered three related major delays that have unexpectedly slowed progress:

1) COVID-19-related delays: The research labs at Johns Hopkins were closed from March 18, 2020 – June 15, 2020. Though this pre-dated this grant period, this resulted in unavoidable delays related to this work:
    a. Upon return to the lab space, restrictions related to person density, equipment availability, and issues with "research re-start" led to lower productivity of lab staff.
    b. In addition, during this lab shutdown, animal colonies – including the TRAMP and hi-Myc colonies to be used in the proposed work – had to be scaled back dramatically, only including breeders necessary for maintaining the lines. It has taken several months to recover these colonies to the point where selection and aging of animals can begin.
    c. Supply chain issues have resulted in difficulty obtaining necessary consumables (most notably pipette tips and tissue culture reagents) have delayed expected expenditure and acquisition of necessary materials for the proposed research.
2) Hiring delays: The lab technologist originally listed in the proposal left for another position in early January 2020. Hiring delays prevented immediate backfilling of this position. The position has been filled as of June 1, 2020, but this project was without a dedicated researcher for 6 months. Due to the applicant pool for the position, the current staff member has substantially less research experience than the previous staff member, with resulting lower productivity. I anticipate that, after a period of training for basic laboratory skills, their productivity to increase. The original pathology fellow listed has left the institution and there was a delay in hiring the current fellow. The position is now filled.
3) Delay in Human Subjects Research approval: Initial IRB approval was delayed due to understaffing / COVID-19-related delays. Approval from the CMRC was further delayed due to PI misunderstanding of necessary paperwork. I anticipate providing all necessary forms by the end of July and apologize for my misunderstanding and the delay. In the interim, the groundwork has been laid for the rapid implementation of the research once approved, including antibody validation, workflow planning, TMA design, etc., that is not reliant on the human specimens themselves.

Changes that had a significant impact on expenditures

No specific changes, but delays in ordering/supply chain and hiring (both due to COVID-19-related effects) have resulted in lower expenditure than anticipated.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents.

Nothing to report.

6. Products

Journal publications

de Groot AE, Myers KV, Krueger TEG, Kiemen AL, Nagy NH, Brame A, Torres VE, Zhang Z, Trabzonlu L, Brennen WN, Wirtz D, De Marzo AM, Amend SR, Pienta KJ. Characterization of tumor-associated macrophages in prostate cancer transgenic mouse models. Prostate. 2021 Jul;81(10):629-647. Published, acknowledgement of federal support

Kuczler MD, Olseen AM, Pienta KJ, Amend SR. ROS-induced cell cycle arrest as a mechanism of resistance in polyaneuploid cancer cells (PACCs). Prog Biophys Mol Biol. 2021 May 12; Epub ahead of print, acknowledgement of federal support

Kostecka LG, Olseen A, Kang K, Torga G, Pienta KJ, Amend SR. High KIFC1 expression is associated with poor prognosis in prostate cancer. Med Oncol. 2021 Mar 24;38(5):47. Published, acknowledgement of federal support

Pienta KJ, Hammarlund EU, Brown JS, Amend SR, Axelrod RM. Cancer recurrence and lethality are enabled by enhanced survival and reversible cell cycle arrest of polyaneuploid cells. Proc Natl Acad Sci U S A. 2021 Feb 16;118(7). Published; acknowledgement of federal support.

Mallin MM, Pienta KJ, Amend SR. Cancer cell foraging to explain bone-specific metastatic progression. Bone. 2020 Dec 3;:115788. Epub ahead of print; acknowledgement of federal support

Pienta KJ, Hammarlund EU, Austin RH, Axelrod R, Brown JS, Amend SR. Cancer cells employ an evolutionarily conserved polyploidization program to resist therapy. Semin Cancer Biol. 2020 Dec 1; Epub ahead of print, acknowledgement of federal support

Pienta KJ, Hammarlund EU, Axelrod R, Brown JS, Amend SR. Poly-aneuploid cancer cells promote evolvability, generating lethal cancer. Evol Appl. 2020 Aug;13(7):1626-1634. Published, acknowledgement of federal support

Lin KC, Sun Y, Torga G, Sherpa P, Zhao Y, Qu J, Amend SR, Pienta KJ, Sturm JC, Austin RH. An in vitro tumor swamp model of heterogeneous cellular and chemotherapeutic landscapes. Lab Chip. 2020 Jul 14;20(14):2453-2464. Published, acknowledgement of federal support

Other publications, conference papers, and presentations.

FRUITS Laboratory, Center for Soft and Living Matter, Institute for Basic Science, Ulsan National Institute of Science and Technology, “Poly-aneuploid Cancer Cells are actuators of universal therapy resistance in cancer”; Ulsan, South Korea

Ecological Society of America Annual meeting, “Optimal foraging of cancer cells promotes lethal phenotype of cancer”; Salt Lake City, UT, USA

Center for Biomarker Discovery & Translation, Div Clinical Chemistry, Johns Hopkins University School of Medicine, “PACCs as actuators of universal therapy resistance”; Baltimore, MD, USA

Inaugural Brady Chairman’s Series, Urology Speaks; The Brady Urological Institute, Johns Hopkins University School of Medicine, “Ecology Meets Cancer Biology: Defining the Keystone Cancer Species to Develop a Cure”; Baltimore, MD, USA

Multiple Myeloma Research Working Group, Indiana University School of Medicine, “Polyaneuploid cancer cells (PACCs): the lynchpin of therapy resistance”; Indianapolis, IN, USA

7. Participants & other collaborating organizations

What individuals have worked on the project?

Name:	Sarah Amend
Project Role:	PI
Researcher Identifier (e.g., ORCID ID)	0000-0002-5606-1262
Nearest person month worked:	2
Contribution to project:	Dr. Amend is the PI of this award and directs all aspects of the work.
Funding Support:	Dr. Amend is supported by this DOD award as well as by NCI P01 (CA093900) and PCF Challenge Award.

Name:	Athen Olseen
Project Role:	Research technologist
Researcher Identifier (e.g., ORCID ID)	
Nearest person month worked:	7
Contribution to project:	Ms Osleen performed PGCC in vitro studies and animal colony management.
Funding Support:	No other support.

Name:	Carolina Gomes Alexandre
Project Role:	Fellow
Researcher Identifier (e.g., ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Dr. Alexandre has supported antibody optimization and workflow development to be implemented upon initiation of pathology work to identify PGCC index.
Funding Support:	No other support.

Name:	Laura Buttitta
Project Role:	Key personnel
Researcher Identifier (e.g., ORCID ID)	0000-0002-5064-0650
Nearest person month worked:	1
Contribution to project:	Dr. Buttitta is a collaborator for this project and is involved in aspects of examining quiescence and cell cycle re-entry in PGCCs.
Funding Support:	Dr. Buttitta is supported by her nine-month appointment in the College of LS&A at the University of Michigan as well as an NIH R01 award from NIGMS (GM127367) and is also Key Personnel on a P01 from NCI (CA093900).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

AMEND, SARAH

18CHAL15
Title: Dissecting the prostate cancer diaspora
Time Commitment: 0.24 calendar months (2% effort)
Supporting Agency: Prostate Cancer Foundation (Pienta, Kenneth - PI)
Name of Procuring Contracting/Grants Officer: Audrey Gardner
Address of Funding Agency: 1250 Fourth Street, Santa Monica, CA 90401
Performance Period: 02/01/19-02/08/22
Level of Funding: $500,000
Project Goal: The primary goal of this project is to determine the rate of CTC production in men with biochemical recurrence and oligometatatic disease in prostate cancer.

Specific Aims:
1: We hypothesize that men in the BCR group will have detectable CTCs if they are in the bone only or LN + bone groups. Only a minority of men with prostate bed or LN only disease will have detectable CTCs.
2: We hypothesize that in men with OM by conventional imaging will have progressed to a systemic disease state and will have detectable CTCs.
Projects overlap or parallel: There is no scientific or budgetary overlap.

W81XWH-20-1-0353
Title: PC190206 Polyploid Giant Cancer Cells actuate prostate cancer therapeutic resistance and lethal phenotype
Time Commitment: 1.8 calendar months (15% effort)
Supporting Agency: CDMRP (Amend, Sarah - PI)
Name of Procuring Contracting/Grants Officer:
Address of Funding Agency:
Performance Period: 4/16/20-3/31/23
Level of Funding: $200,000
Project Goal: The primary goal of this study is to investigate the role of PGCCs in prostate cancer therapy resistance and the development of the lethal prostate cancer phenotype.
Specific Aims:
1: Determine how and when PGCCs are formed during prostate cancer progression.
2: Demonstrate that quiescence underlies a common mechanism of therapy resistance in PGCCs.
Projects overlap or parallel: There is no scientific or budgetary overlap.

SUBK00011358 (P01CA093900)
Title: The Biology of Prostate Cancer Metastasis
Time Commitment: 1.2 calendar months (10% effort)
Supporting Agency: Subaward from University of Michigan / Prime award NCI (Pienta, Kenneth - PI)
Name of Procuring Contracting/Grants Officer: Dean Michalak
Address of Funding Agency: 3003 S. State Street, Ann Arbor, MI 48109-1287
Performance Period: 07/02/2020-05/31/2025
Level of Funding: $777,465
Project Goal: The primary goal of this study is to investigate how PGCCs mediate prostate cancer metastasis.
Specific Aims:
1: Determine when and how metastasis-initiating PGCCs are formed.
2: Determine that senescence is a mechanism of stress resistance in PGCCs.
3: Demonstrate that PGCCs are important initiators of metastasis.
Projects overlap or parallel: There is no scientific or budgetary overlap.

BUTTITTA, LAURA

Title: Chromatin remodeling at cell cycle exit
Time Commitments: 1 SM
Supporting Agency: NIH
Address:
45 Center Drive MSC 6200
Bethesda, MD 20892-6200
Contracting/Grants Officer:
Anthony Carter, Ph.D.
Program Director
NATIONAL INSTITUTE OF GENERAL MEDICAL SCIENCES
carteran@nigms.nih.gov
301-496-7301
Performance period: 08/01/2018-07/31/2023
Level of funding: $1,586,191
Project Goals: States of cellular withdrawal from the cell cycle or G0 can range from readily reversible to permanently postmitotic. This proposal addresses how different states of G0 are controlled during development and why some are more reversible than others. This work has the potential to impact a wide range of biological

questions, as the proper control of G0 is critical for proper development and during tissue regeneration, but becomes disrupted in cancer.
Specific Aims:
Aim 1: Determine which changes in chromatin accessibility are due to cell cycle exit
Aim 2: Determine how developmental signals coordinate cell cycle exit with differentiation.
Aim 3: Examine the role of nucleosome remodelers in promoting cell cycle exit.
Overlap: None

Title: Biology of Prostate Cancer Skeletal Metastases
Time Commitments: 0.06 SM and 0.9 AY
Supporting Agency: NIH
Address:
Division of Cancer Biology
9609 Medical Center Drive MSC 9747/9748
Bethesda, MD 20892
Contracting/Grants Officer:
WATSON, JOANNA M
Program Officer
National Cancer Institute
watsonjo@mail.nih.gov
(240) 276-6220
Performance period: 07/01/2020 – 03/31/2025
Level of funding: $45,220
Project Goals: The goals of the Buttitta portion of this project are to 1. Identify roles for polyploid giant cancer cells in mediating prostate cancer metastasis and resistance to treatment and 2. Examine roles for Abscissic Acid signaling in dormancy of prostate cancer in the bone.

Specific Aims:
Project 1 explores the novel finding that chemotherapy induces fusion of PCa cells to form multinuclear polyploid giant cancer cells (PGCCs) that confer chemoresistance in the bone microenvironment; Project 2 examines the exciting idea that abscisic acid (ABA) induces PCa cells to adopt a phenotype capable of existing in a dormant and chemoresistant state, with the capacity for long-term survival and potential to develop into overt bone metastases; Project 3 explores the surprising role that osteocytes (OCys) play in promoting PCa bone metastasis through activation of a novel growth differentiation factor-15 (GDF15) receptor, GDFN family receptor alpha-like precursor (GFRAL), that subsequently promotes PCa metastatic invasion and growth; Project 4 investigates the novel hypothesis that macrophage efferocytosis (engulfment) of apoptotic PCa cells induces immunosuppressive signaling in the bone microenvironment that subsequently enhances metastatic growth.

Overlap: None

DE MARZO, ANGELO

ACTIVE
P50CA58236 (De Marzo, PI of Core B)
Title: SPORE in Prostate Cancer, Core B – Specimen Core
Effort: 1.20 calendar (10%)
Supporting Agency: NIH/NCI
Name of Contracting/Grants Officer: NIH/NCI Grants Associate- AMO
Address of Funding Agency: NIH 616 Executive Boulevard, Suite 7013, MSC 8347, Rockville, MD 20852/ NCI Public Inquires Office 6116 Executive Boulevard Room 3036A Bethesda, MD 20892-8322
Performance Period: 9/25/2014-08/31/2021
Level of Funding: $63,124
Project's Goal/Specific Aims: The major goals of this Core are to maintain and enhance a repository of prostate tissues containing a wide range of neoplastic and non-neoplastic samples from both fresh frozen and paraffin blocks, prostatic fluids, DNA, RNA, and protein, to formalize standard workflows, operating and quality

control policies and procedures for the collection, storage to distribute these samples to SPORE and other investigators as needed, and to perform innovative biospecimen research using these specimens.
Aim 1: To maintain and enhance a repository of prostate tissues containing a wide range of neoplastic and non-neoplastic samples from both fresh frozen and paraffin blocks, prostatic fluids, DNA, RNA, and protein, and, to distribute these samples to SPORE and other investigators as needed.
Aim 2: To provide high quality histopathologic diagnoses of tissue specimens and tissue microarrays.
Aim 3: To perform well-controlled immunohistochemistry (IHC) assays, interpretation and quantitative analyses of IHC slides to facilitate the achievement of the specific aims of the individual research projects.
Aim 4: To continue to design, produce and distribute tissue microarrays using human prostate tissues, cell lines, and xenografts.
Aim 5: To continue to improve and add tools to our open source tissue microarray database and software (TMAJ) (http://tmaj.pathology.jhmi.edu) including the development and dissemination of new open source image analysis tools "FRIDA" (FRamework for Image Dataset Analysis) while ensuring compatibility with the CaBIG grid system.
Aim 6: To test and potentially implement emerging software tools, such as CaTissue, from the CaBIG program for specimen banking efforts, serving as a model for other SPOREs and other research programs throughout our University.
Aim 7: To provide a facility and pathology expertise for laser capture microdissection.
Aim 8: To continue to function as the Central Pathology Core for the Inter-Prostate SPORE Biomarker Study (IPBS).
Projects overlap or parallel: No scientific or budgetary overlap

U01CA196390 (Pienta/De Marzo – Co-PIs)
Title: Multidisciplinary Integrative Genomic Approach to Distinguish Lethal from Indolent Prostate Cancer in Men of European and African Ancestry
Effort: 1.2 calendar (10%)
Supporting Agency: NIH
Name of Contracting/Grants Officer: Elizabeth Woodhouse
Address of Funding Agency: 9000 Rockville Pike, Bethesda, MD 20892
Performance Period: 09/10/2015-08/31/2021
Level of Funding: $495,653(NCE)
Project's Goal/Specific Aims: The overall goal of this project is to develop a distinctive molecular signature that can predict the subsequent fates of early lesions distinguishing indolent from progressive disease with lethal potential.
Aim 1: Develop Integrated Genomic, Epigenomic and Expression Profiling Signatures of Indolent and Aggressive Prostate Cancer from both White and African-American Men.
Aim 2: Validate Biomarkers and Pathways in Active Surveillance and Autopsy Patients.
Aim 3: Validate Biomarkers in Relation to Patient Outcome, with Emphasis on Intermediate Risk and African American Patients.
Projects overlap or parallel: No scientific or budgetary overlap

ICD 856497 (De Marzo)
Title: Can High Grade PIN Be Distinguished from Intra-Acinar Spread of Prostatic Carcinoma? Implications for Chemoprevention Trials
Effort: 0.60 calendar (5%)
Supporting Agency: Janssen Research & Development, LLC
Name of Contracting/Grants Officer: Kathy Zelinsky
Address of Funding Agency: 1400 McKean Road, Office 32-22528, Spring House, PA 19477
Performance Period: 12/23/2015-12/31/2021
Level of Funding: $1,786,130 (NCE)
Project's Goal/Specific Aims: The goal of this research project is to determine if high grade PIN can be distinguished from intra-acinar spread of prostatic carcinoma.
Aim 1: To use whole genome-based next-generation DNA sequencing, RNAseq, and MBDseq on well-characterized fresh-frozen human pathology specimens from prostatectomies to determine which PIN lesions most likely represent intraductal spread of existing carcinoma and which most likely represent precursor lesions

Aim 2: To develop a marker-based approach to help determine, on a given prostatic needle biopsy, whether a histologically evident PIN lesion is actually a potential precursor or represents already invasive carcinoma that has spread in a retrograde manner.
Projects Overlap or Parallel: No scientific or budgetary overlap

P30CA006973 (Nelson; Co-PI of Core)
Title: Regional Oncology Research Center
Effort: 0.60 calendar (5%)
Supporting Agency: NIH/NCI
Name of Contracting/Grants Officer: Jason Gill
Address of Funding Agency: 9000 Rockville Pike Bethesda M109644168D, 20892
Performance Period: 05/07/2017-04/30/2022
Level of Funding: $100,000
Project's Goal/Specific Aims: Provide a centralized Core facility, including administrative, technical and logistical support for performing rapid autopsies on cancer patients succumbing to a wide range of tumor types. This includes patients in ongoing clinical trials who are pre-identified and consented by SKCCC investigators for correlation of molecular markers with clinical trial outcomes, as well as consenting patients not enrolled in clinical trials.
Projects overlap or parallel: No scientific or budgetary overlap

W81XWH-17-1-0286 (Sfanos)
Title: PC160336-Interaction Between the Inflammatory Microenvironment and Somatic Genomic Alterations as a Driver of Prostate Cancer Aggressiveness in African American Men
Effort: 0.6 calendar (5%)
Supporting Agency: US Army Research Council
Name of Contracting/Grants Officer: Kimberly Carter
Address of Funding Agency: DOD Prostate Cancer Research Program, CDMRP, U.S. Army Medical Research and Materiel Command MCMR-CD, 1077 Patchel Street, Fort Detrick MD 21702-5024; telephone: 301-619-7782; fax: 301-619-7796
Performance Period: 07/01/2017-06/30/2021
Level of Funding: $166,899(NCE)
Project's Goal/Specific Aims:
Aim 1: To characterize the immune cell components and degree of inflammation in the prostate tumor microenvironment in tumors from AA and WH men and determine whether specific tumor immune cell profiles are associated with biochemical recurrence after radical prostatectomy in either population.
Aim 2: To test whether specific tumor immune profiles are associated with underlying tumor somatic genomic alterations in AA and WH men and determine whether there is an interaction between immune microenvironment and genomic alterations in the association with oncologic outcomes in AA men and in WH men.
Projects overlap or parallel: No scientific or budgetary overlap

W81XWH-17-1-0425 (Lotan)
Title: PC160783-Prospective-Retrospective Analysis of PTEN Immunohistochemistry Assay for Prediction of Outcomes in Recurrent and Metastatic Prostate Cancer
Effort: 0.24 calendar (2%)
Supporting Agency: Department of the Army
Name of Contracting/Grants Officer: Janet Kuhns
Address of Funding Agency: 820 Chandler Street, Fort Detrick, MD 21702-5014
Performance Period: 09/01/2017-09/29/2020
Level of Funding: $600,00 (NCE)
Project's Goal/Specific Aims: The major goal of this project is to examine association of PTEN loss and ERG status with outcomes in two large clinical trials for advanced/metastatic prostate cancer (RTOG 9601 and ECOG 3805).
Projects overlap or parallel: No scientific or budgetary overlap

109644168 (Lotan)
Title: Core C: Pathology, Biospecimen and Immune Profiling Core
Effort: 0.60 calendar (5%)

Supporting Agency: University of Southern California
Name of Contracting/Grants Officer: Lillian Rivera
Address of Funding Agency: 2001 Soto Street, SSB-205, Los Angeles, CA 90089-9235
Performance Period: 07/05/18-08/31/22
Level of Funding: $165,060
Project's Goal/Specific Aims: The major goal of this project is to assemble a prospective cohort of African-American prostate cancer patients from SEER registries across the country. Dr. Lotan will lead the Pathology Core for this project, processing ~3000 prostate cancer tumor specimens from this cohort.
Project overlap or parallel: No scientific or budgetary overlap

W81XWH-18-2-0013 (Sfanos)
Title: PC171113-Prostate Cancer Biorepository Network (PCBN)
Effort: 0.60 calendar (5%)
Supporting Agency: CDMRP
Name of Contracting/Grants Officer: Nrusingha Mishra
Address of Funding Agency: DOD Prostate Cancer Research Program, CDMRP, U.S. Army Medical Research and Materiel Command MCMR-CD, 1077 Patchel Street, Fort Detrick MD 21702-5024
Performance Period: 09/30/18-09/29/21
Level of Funding: $225,000
Project's Goal/Specific Aims: This project will coordinate among four institutions the development of a prostate cancer bio-repository with well-annotated specimens obtained using optimized and standardized protocols, and to conduct biospecimen science to characterize critical factors influencing the molecular integrity of research tissues.
Project overlap or parallel: No scientific or budgetary overlap

W81XWH-18-2-0015 (De Marzo)
Title: PC171113P1-Prostate Cancer Biorepository Network (PCBN) – Johns Hopkins Network
Effort: 1.2 calendar (10%)
Supporting Agency: CDMRP
Name of Contracting/Grants Officer: Nrushinghas C. Mishra, PhD
Address of Funding Agency: US Army Medical Research & Materiel Command, 820 Chandler Street, Fort Detrick, MD 21702-5014
Performance Period: 09/30/18-09/29/21
Level of Funding: $450,000
Project's Goal/Specific Aims: The goal of the PCBN is to develop a biorepository with high quality, well-annotated specimens obtained in a systematic, reproducible fashion using optimized and standardized protocols, and an infrastructure to facilitate the growth of the resource and its wide usage by the prostate cancer research community. The PCBN will also conduct and support biospecimen science that characterizes critical factors influencing the molecular integrity of research tissues.
Project overlap or parallel: No scientific or budgetary overlap

W81XWH-19-1-0511 (Shenderov)
Title: PC180806 Understanding Antitumor T cell Immunity in High-Risk Localized Prostate Cancer after Neoadjuvant Treatment with an Antibody Targeting the B7-H3 Checkpoint
Effort: 0.24 calendar (2%)
Supporting Agency: CDMRP
Name of Contracting/Grants Officer: Kimberly Carter
Address of Funding Agency: USA Med Research ACQ Activity, 820 Chandler Street, Fort Detrick, MD 21702-5014
Performance Period: 08/01/19-07/31/23
Level of Funding: $161,450
Project's Goal/Specific Aims: To perform a first-in-man biological and immunological characterization of how B7-H3 targeted therapy affects outcomes in men with high-risk prostate cancer utilizing clinically annotated samples from a soon-to-be completed phase II clinical study (NCT02923180).
Aim 1: To determine whether enoblituzumab results in pathologic anti-tumor responses by evaluating tumor cell apoptosis and tumor microenvironment (TME) T cell infiltrates pre- and post-treatment
Aim 2: To analyze targetable immune checkpoints adaptively induced upon enoblituzumab treatment.

Aim 3: To analyze the repertoire and gene-expression profiles of tumor-specific T cells pre- and post-enoblituzumab treatment.
Project overlap or parallel: No scientific or budgetary overlap

90082514 (Antonarakis)
Title: Concurrent Administration of Bipolor Androgen Therapy (BAT) and Nivolumab in Metstatic Castration-Resistant Prostate Cancer: The COMBAT-CRPC Trial
Effort: 0.30 calendar (2.5%)
Supporting Agency: PCF
Name of Contracting/Grants Officer: Howard Soule
Address of Funding Agency: 1250 Fourth Street, Santa Monica, CA 90401
Performance Period: 02/28/19-02/28/22
Level of Funding: $500,000
Project's Goal/Specific Aims: The major goal of this research is to test the hypothesis that a benefit of immunotherapy may be observed in mCRPC patients with germline and/or somatic mutations in DNA-repair genes, including homologous recombination genes (i.e. not just MMR genes), thus potentially expanding the pool of metastatic CRPC patients that may be susceptible to immune checkpoint blockade.
Aim 1: Conduct a Biomarker-Rich Phase II Trial of BAT in Sequence with Nivolumab for mCRPC Patients.
Aim 2: Evaluate the Role of "Nucleophagy" as a Means of Clearing BAT-induced dsDNA Breaks, and Assess the Contribution of Cytoplasmic DNA in Activating Innate and Adaptive Immune Responses.
Aim 3: Assessment of DNA Damage Markers, Immune Cell Infiltration, and Generation of Novel Mutation-Associated Neoantigens (MANAs) from Tumor Biopsy Specimens.

PAR-18-290 (Pienta)
Title: The Biology of Prostate Cancer Skeletal Metastases
Effort: 0.36 calendar (3%)
Supporting Agency: Subaward from University of Michigan
Address of Supporting Agency: 5071 Wolverine Tower, 3003 S. State St., Ann Arbor, MI 48109-1287
Name of Grants Officer: Dean Michalak
Performance Period: 06/01/20-05/31/25
Level of Funding: $106,600
Project Goal: The overall goal of this study is to investigate to the role PGCCs play in tumorigenesis, skeletal metastasis, and therapeutic resistance of prostate cancer.
Aim 1: Determine how and when PGCCs are formed.
Aim 2: Demonstrate that reversible senescence underlies a common mechanism of stress resistance in PGCCs.
Aim 3: Demonstrate that PGCCs are important initiators of metastasis.
Overlap: No scientific or budgetary overlap.

R01CA255349-01 (Platz, Meeker, MPIs)
Title: Stromal Senescence in Lethal Prostate Cancer: A Novel Target for Prognosis and Therapy
Effort: 0.3 calendar months (2.5%)
Supporting Agency: NIH/NCI
Address of Supporting Agency: 6116 Executive Boulevard, Suite 7013, MSC 8347, Rockville, MD 20852
Name of Grants Officer: TBD
Performance Period: 09/01/2020-08/31/2024
Level of Funding: $393,259
Project Goals: Goal 1 is to inform the pressing clinical need for identifying which men's prostate cancers are very likely to kill and, equally important, which ones are very unlikely to kill. If our hypothesis is confirmed, information from our work could be incorporated into prognostic tools. Goal 2 is to inform use of novel therapeutics that eliminate senescent stromal fibroblasts in men at risk for progression or harboring metastases.
Aim 1. Evaluate the association between senescent stromal fibroblasts, especially in the presence of stromal inflammation, in prostatectomy tissue and risk of progression to metastatic prostate cancer in men with intermediate and high-risk disease (Cohort 1). In this case-cohort study, men who received hormone, chemo-, or radiation therapy before or after surgery but before the detection of metastasis by imaging were excluded to be able to address how biomarkers are associated with outcome without interference by treatment subsequent to prostate removal during follow-up.

Aim 2. Evaluate the association between senescent stromal fibroblasts, especially in the presence of stromal inflammation, in prostatectomy tissue and risk of progression to metastasis or rapidly rising PSA in a second, independent cohort of men with intermediate and high-risk disease (Cohort 2). This case-cohort study also excludes men treated before surgery, but differs from Cohort 1 by retaining men treated after surgery, but before detection of metastasis, and by including an earlier lethal event (rapidly rising PSA). If our hypothesis is confirmed, we will determine prognostic performance of senescent stromal fibroblasts, and assess whether their addition enhances performance of existing, but imperfect cancer cell-based genomic prognostic tests. We expect that this stromal biomarker (reflecting tumor microenvironment) will be complementary to cancer-based biomarkers. The Decipher test (GenomeDx) has been evaluated in Cohort 1 and the Prolaris test (Myriad Genetics) has been evaluated in Cohort 2.
Aim 3. Determine whether senescent fibroblasts are present in metastases, and if so, their heterogeneity across metastatic sites of bone and soft tissues in men who died of castrate-resistant prostate cancer. We will use a castration-resistant metastatic prostate cancer rapid autopsy series and associated metastatic tissue from multiple lesions from a variety of soft tissues and bone per man.
Role: Co-investigator
Overlap: There is no overlap between application R01CA255349-01 under consideration and the funded DOD grant W81XWH-19-PCRP-IDA. While both address the topic of stromal senescence in the prostate, the points in the natural history of prostate cancer to be investigated and study samples to be used are distinct.

90084252 (De Marzo)
Title: Genomic studies of aggressive prostate cancer lesions
Effort: 0.36 calendar (3%)
Supporting Agency: Schwab Charitable Fund
Name of Contracting/Grants Officer: N/A
Address of Funding Agency: P.O. Box 628298 Orlando, FL 32862
Performance Period: 07/01/19 – 06/30/21
Level of Funding: $207,230 (NCE)
Project's Goal/Specific Aims: To shed light onto prostate cancer dormancy and anoikis resistance, our proposal seeks to identify molecular and environmental mechanisms through which cribriform lesions develop to improve our understanding of the aggressive lethal and metastatic phenotype.
Aim 1-2: Characterization of the 1) RNA transcriptome and 2) somatic genome, of separate compartments in cribriform glands.
Aim 3: Prospective collection of prostate cancer samples from patients with metastatic castration resistant prostate cancer.
Projects overlap or parallel: No scientific or budgetary overlap

W81XWH-20-1-0353 (Amend)
Title: Polyploid Giant Cancer Cells Actuate Prostate Cancer Tumor Resistance and Lethal Phenotype
Time Commitment: 0.24 calendar months (2% effort)
Supporting Agency: CDMRP
Name of Procuring Contracting/Grants Officer: Joshua McKean
Address of Funding Agency: 820 Chandler St., Fort Detrick, MD 21702-5014
Performance Period: 04/01/20-03/31/23
Level of Funding: $201,269
Project Goal/Specific Aims: The primary goal of this project is to better understand how PGCCs play a role in cancer treatment resistance and the lethal phenotype of prostate cancer.
Aim 1: Determine how and when PGCCs are formed during prostate cancer progression.
Aim 2: Demonstrate that quiescence underlies a common mechanism of stress resistance in PGCCs.
Projects overlap or parallel: None

W81XWH-20-1-0264 (Platz)
Title: Evaluating senescent stromal fibroblasts as a promoter of prostate cancer lethality to inform a paradigm shift in prognostic, predictive, therapeutic strategies
Effort: 0.24 calendar (2%)
Sponsor: CDMRP
Name of Contracting/Grants Officer: TBD
Address of Funding Agency: 820 Chandler St., Fort Detrick, MD 21702-5014
Performance Period: 07/01/2020-06/30/2023

Level of Funding: $259,295
Project's Goal/Specific Aims: Our goal is to determine whether senescent prostate stromal fibroblasts, especially those with the senescence-associated secretory phenotype (SASP), promote lethal prostate cancer, and are more prevalent in Black men.
Aim 1: For Aim 1, in pre-diagnostic (benign) prostate biopsy tissue with risk of prostate cancer in men at risk. We will generate a case-cohort study (320 subcohort) sampled from a prospective cohort of 2,584 men from the PCPT placebo arm without clinical indication who underwent end-of-study biopsy per trial protocol and negative for prostate cancer by linkage with claims data up to 17 years later. Use of these biopsies as baseline provides an epidemiologically sound, temporally correct approach.
Aim 2: For Aim 2, in benign and prostate tumor tissue with risk of progression to lethal prostate cancer in men surgically- treated for clinically localized prostate cancer. We will use a PCBN nested case-control study with TMAs developed for testing biomarkers of prostate cancer progression (426 progressors, 426 matched controls). Also, we will evaluate if prevalences of these fibroblasts and associations with lethal disease differ between the tumor microenvironment and benign tissue.
Aim 3: For Aim 3, in benign and prostate tumor tissue with risk of further progression to metastasis or prostate cancer death in men surgically-treated and who received salvage therapy for PSA recurrence. We will design a propensity score matched cohort study of 392 men who received salvage RT ±AAT after rising PSA post-prostatectomy and construct TMAs. No such study exists in the PCBN. Also, we will evaluate if these fibroblasts are predictive of response to salvage RT ±AAT versus salvage RT only.
Aim 4: In Aim 4, we will determine whether the phenotypic nature, density, and distribution of senescent stromal fibroblasts in benign and prostate tumor tissue differ between matched Black and White men with prostate cancer. We will use TMA sets in the PCBN for Black (N=135) and White (N=135) men matched on clinicopathologic characteristics that were designed to investigate racial differences in prostate biomarkers.
Projects overlap or parallel: No overlap with the current application.

19CHAS03 (Sfanos)
Title: The Inflammatory Microenvironment of Aggressive Prostate Cancer in African American Men (Project 4/RESPOND)
Effort: 0.24 calendar (2%)
Supporting Agency: Prostate Cancer Foundation
Name of Contracting/Grants Officer: Howard Soule
Address of Funding Agency: 1250 Fourth Street, Santa Monica, CA 90401
Performance Period: 10/11/2019-10/10/2024
Level of Funding: $200,000
Project's Goal/Specific Aims: This project will leverage ongoing collaborations with the newly NIH funded RESPOND study that over the next five years will recruit 10,000 African American men with prostate cancer from across the U.S. with a focus initially on identifying social and biological factors that may be linked with the development of aggressive prostate cancer, recurrence and survival. By characterizing and quantifying the immune cell subsets in a large number of AA men in the RESPOND cohort, we aim to distinguish an immune cell profile associated with aggressive disease that is more common in AA men.
Projects overlap or parallel: No scientific or budgetary overlap

PA-19-056(Brennen)
Title: A First-in-Class FAP-activated Protoxin to disrupt the Tumor-Stroma Parasitic Cycle fueling lethal Prostate Cancer Progression
Effort: 0.60 calendar (5%)
Supporting Agency: NIH
Address of Supporting Agency: 9000 Rockville Pike Bethesda M109644168D, 20892
Name of Grants Officer: Justin Birkin
Performance Period: 01/01/21-12/31/25
Level of Funding: $310,093
Project Goal: The overarching goal of this proposal is to develop a FAP-targeted therapy to selectively eliminate tumor-infiltrating FAP+ cells (i.e. TAMs, MSCs, CAFs); concurrently priming immune recognition of malignant PCa and disrupting the tumor-stroma "parasitic cycle" fueling tumor growth.
Aim 1: Comprehensive assessment of FAP expression, immunologic profile, and outcome in a unique series of human PCa tissues including pelvic lymph nodes and distant metastases.
Aim 2: Evaluate efficacy, toxicity, and pharmacokinetic (PK) parameters of FAP-activated niclosamide in a novel series of human PCa patient-derived xenografts (PDXs) obtained from men with lethal disease.

Aim 3: Evaluate efficacy and immunologic impact of targeting FAP-positive cells using an enzymatically-activated mitochondrial protoxin in syngeneic models of lethal PCa.
Overlap: No scientific or budgetary overlap.

90088420 (Yegnasubramanian)
Title: Development of Assays for Detection of Methylated DNA to be performed on the GeneXpert platform
Effort: 0.12 calendar months (1%)
Supporting Agency: Cepheid
Name of Contracting/Grants Officer: Scott A. Campbell
Address of Funding Agency: 904 Caribbean Drive, Sunnyvale, CA 94089
Performance Period: 05/01/20-10/31/21
Level of Funding: $85,714 (NCE)
Project Goals: To develop a new technology for assessment of DNA methylation alterations in prostate cancer.
Overlap: No overlap with the current application

PC200308 (Trock)
Title: PC200308 Metabolomic and Methylation Pathways Associated with Black-White Disparity in Lethal Prostate Cancer
Effort: 0.60 calendar months (5%)
Supporting Agency: CDMRP
Name of Contracting/Grants Officer: TBD
Address of Funding Agency: U.S. Army Medical Research and Materiel Command MCMR-CD, 1077 Patchel Street, Fort Detrick, MD 21702-5024
Performance Period: 07/01/21-06/30/24
Level of Funding: $749,907
Project's Goals/Specific Aims: Our hypothesis is that the racial disparity in PCa development and outcomes between AA and EA men is partially due to metabolomics and epigenetic modifications influencing inflammation, lipid, energy, and one-carbon pathways.
Aim 1: Construct a matched case-control population of AA men with vs. without BCR, and EA men with vs. without BCR, with cases and controls matched on age and year of prostatectomy and AA and EA also matched on age and year of prostatectomy.
Aim 2: Aliquot prospectively collected previously banked prostatic fluid from the 2 matched case-control sets, and submit to Metabolon, Inc. for global metabolomics analysis, and to the laboratory of Dr. Yegnasubramanian for epigenome-wide analyses.
Aim 3: Conduct biostatistical/bioinformatics analyses of metabolomics and epigenetics data, including pathway analyses focusing on lipid, inflammation, energy, and one-carbon pathways to evaluate how the metabolomics and epigenetics of BCR differ between AA and EA men.

W81XWH-21-1-0295 (Nelson)
Title: GSTP1-Positive Subset of Prostate Cancer Over-Represented in African-American Men: Systemic Treatment Implications
Effort: 0.60 calendar months (5%)
Supporting Agency: CDMRP
Name of Contracting/Grants Officer: Kimberly Carter
Address of Funding Agency: 820 Chandler St., Fort Detrick, MD 21702-5014
Performance Period: 05/01/21-04/30/24
Level of Funding: $1,228,125
Project's Goals/Specific Aims: To test the hypothesis that GSTP1-positiveprostate cancers comprise a distinct disease subset, with a discrete phenotype, that is more prevalent in AA men than EA men, and that affects responses to taxane chemotherapy.
Aim 1: Molecular characterization of GSTP1-positiveprostate cancers, using genome, epigenome, and transcriptome sequencing.
Aim 2: Ascertain the mechanistic implications of persistent GSTP1 expression, especially persistent GSTP1-I105V expression, for responses of prostate cancers to taxanes used in the treatment of life-threatening prostate cancer.
Aim 3: Determine the natural history of GSTP1+ prostate cancers, including the propensity to progress to life-threatening disease.

Overlap: No scientific or budgetary overlap.

What other organizations were involved as partners?

Nothing to report.

8. Special reporting requirements

N/A

9. Appendices

Enclosed pertinent published manuscripts.

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Cancer cells employ an evolutionarily conserved polyploidization program to resist therapy

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ABSTRACT

Unusually large cancer cells with abnormal nuclei have been documented in the cancer literature since 1858. For more than 100 years, they have been generally disregarded as irreversibly senescent or dying cells, too morphologically misshapen and chromatin too disorganized to be functional. Cell enlargement, accompanied by whole genome doubling or more, is observed across organisms, often associated with mitigation strategies against environmental change, severe stress, or the lack of nutrients. Our comparison of the mechanisms for polyploidization in other organisms and non-transformed tissues suggest that cancer cells draw from a conserved program for their survival, utilizing whole genome doubling and pausing proliferation to survive stress. These polyaneuploid cancer cells (PACCs) are the source of therapeutic resistance, responsible for cancer recurrence and, ultimately, cancer lethality.

1. Introduction

Metastatic cancer remains unbeatable. Metastatic cancer eventually becomes resistant to all therapies and kills more than 10 million people per year globally [1–3]. It is generally accepted that the malignant cells of a tumor evolve. This means that multiple genetically distinct subclones of cancer cells that originated from a single initiating cancer cell all exist in the tumor(s) of a single patient, resulting in high cancer cell genetic heterogeneity [4–25]. This genetic heterogeneity is generally accepted as the root of therapeutic resistance: a cell lineage resistant to a class of therapy occurs from random and chance genetic mutation (Fig. 1). We have recently observed that it is likely that resistance is an example of convergent evolution leading to lethal cancer [3]. We believe that resistance is mediated through ecological and evolutionary properties of cancer cells that enter a cell-state transition that includes 1) polyploidization of their aneuploid genome, and 2) exiting of the cell cycle to pause proliferation, forming polyaneuploid cancer cells (PACCs). After stress is removed, PACCs undergo depolyploidization to repopulate the tumor, representing the source of the "rescue effect" associated with the catastrophic event of therapeutic intervention [26].

Here, we place PACCs in the context of polyploidy found in single-celled and multi-cellular organisms. Variously across the tree of life, polyploidy may provide a state that serves both ecological and evolutionary functions. We discuss how, ecologically, polyploid cells may have 1) high survivorship under harsh conditions, 2) higher capacity for producing RNA and protein products, and 3) higher capacity for nutrient uptake. Evolutionarily, polyploid cells may 1) enhance DNA repair, 2) counteract Muller's ratchet, 3) generate heritable variation among their offspring, and 4) permit self genetic modification in which the polyploid cell is able to create progeny that possess a heritable solution to a stressor that threatens the viability of the population.

2. Polyaneuploid Cancer cells

Large polymorphous cancer cells have been described by physicians and scientists since the 1850′s (Fig. 2) [27–58]. The majority of the

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cancer research and treatment development communities have disregarded these cells as irreversibly senescent or destined for mitotic catastrophe and death. A small number of pioneering scientists, including Erenpreisa, Cragg, Illidge, Liu, Walen, Rajaraman, Mirzayans and their colleagues, have now made it clear that these cells - most commonly termed polyploid giant cancer cells (PGCCs), but also referred to as multinucleated giant cancer cells, blastomere-like cancer cells, osteoclast-like cancer cells, pleomorphic cancer cells, large cancer stem cells, and polyaneuploid cancer cells (PACCs) - are important mediators of tumorigenesis, metastasis, and therapeutic resistance. Virtually all cancer cells are aneuploid (having an abnormal number of chromosomes or parts of chromosomes), and this aneuploidy is unique from tumor-to-tumor and cancer cell lineage-to-lineage [54]. PACCs are formed when these aneuploid cells undergo whole genome doubling in response to stress, resulting in multiple full sets of their cancer cell lineage's aneuploid genome, i.e., polyaneuploidy [26]. PACCs are present as a minor component of cell lines from all tumor types examined to date (Fig. 2A). They are also present in patients with nearly all types of metastatic cancer (Fig. 2B).

The totality of data supports a hypothesis that therapeutic resistance arises as a result of the emergence of PACCs within a population of cancer cells (Fig. 3). PACCs appear to be a reversible phase in the life cycle of lethal cancers, i.e., a life history cell-state. They form as a result of cancer cells' response to tumor microenvironment stress that accesses evolutionary and developmental programs for polyploidy, resulting in whole genome doubling (WGD) of the aneuploid genomic complement, increased cell size, and increased cell contents. PACC formation results in a cancer cell phenotype of reversible cell cycle arrest to avoid DNA damage, providing a mutation agnostic universal mechanism of therapeutic resistance. The increased cell size associated with WGD and the pause in cell cycling allows for the production of cellular machinery to cope with stress, increased intracellular nutrients to survive quiescence, and increased genetic material to allow for both genome stability in the short term and access to increased heritable variation over time. After a therapeutic stress, PACCs exhibit the ability to re-enter the cell cycle and undergo depolyploidization to repopulate the tumor with resistant non-polyploid progeny that then make up the bulk of cancer cells within a tumor [27,51,55,59–62].

3. Accessing evolutionary and developmental polyploid programs is a critical step in PACC formation

Resilience to environmental perturbations through WGD and concomitant cell enlargement has been documented across numerous taxa as convergent stress response programs, including prokaryotes (archaea and bacteria), unicellular eukaryotes, and multicellular plants and animals [63–67]. These evolutionary stress response programs are reflected in the developmental programs of human tissue as normal cells respond to physiologic needs and stress. The ability to form PACCs in response to stress, therefore, appears to be a by-product of the convergent polyploid program utilized by noncancerous organisms and cells (Fig. 4). Once accessed, cancer cells can use this program to survive and react to tumor microenvironmental stresses as well as extrinsic therapeutic stresses [68–70].

There are a limited number of ways by which a cell can become polyploid. Importantly, in both the initial generation of and during the life of polyploid cells, DNA replication, karyokinesis, and cytokinesis are not necessarily linked. In addition to cell fusion, polyploidy can be generated through endocycling, mitotic slippage, or endomitosis (Fig. 5) [71–77]. Terminology surrounding the generation of polyploidy can be somewhat confusing, with multiple overlapping terms. Endocycling, also referred to as endoreplication and endoreduplication, is the replication of DNA in S-phase without the cell entering mitosis. This results in a single nucleus with increased ploidy (e.g., 4 N). Mitotic slippage occurs when a cell exits the division cycle just prior to anaphase at the spindle assembly checkpoint [71]. This also results in a single nucleus. Endomitosis, also referred to as cytokinesis failure, occurs during anaphase, resulting in a single nucleus, or in telophase, resulting in a multiple nuclei within a single cell [40,76,78–80]. The mitotic cycle and the exits resulting in polyploidization are tightly regulated through multiple checkpoints that are beyond the scope of this discussion [78,81–90]. Polyploid programs provide increased fitness on evolutionary timescales (across species) as well as within the lifespans of individual organisms (across tissues) [63,74,76,91–93]. The evolutionary programs are engaged in response to environmental perturbations and form the basis for the developmental programs that are observed in specialized tissues in response to extrinsic stresses or metabolic requirements (Tables 1 and 2). These evolutionary and developmental programs demonstrate how polyploidy is adaptive and improves fitness of PACCs.

4. Evolutionary programs for polyploidy across the tree of life

As we explore the presence, mechanisms, and function of polyploid cells across diverse taxa, there are several recurrent themes. First there are ecological advantages to large polyploid cells in terms of surviving stress, proliferative cell cycle arrest, and increased metabolic potential. Second, there are evolutionary advantages in terms of gene repair and opportunities for accelerating rates of evolution. In the following sections we shall explore diverse taxa and evaluate them in terms of the ecological and evolutionary advantages afforded by polyploidy as well as the mechanisms for polyploidization.

5. Archaea

Polyploidy in prokaryotes is reflected in the duplication of the haploid single circular chromosome [94–96]. Multiple advantages of increased genomic material with accompanying increase in cell size have been proposed. Non-genetic advantages include how an increased cell size may facilitate predator avoidance or increase foraging rates and ability [97,98]. At least one species, *Haloferax volcanii*, appears to use genomic DNA as a storage mechanism for phosphate that can be used to synthesize important building blocks including membranes, DNA, and protein [99]. Multiple copies of genes may allow the cell to produce more of the gene product more rapidly. The presence of multiple copies

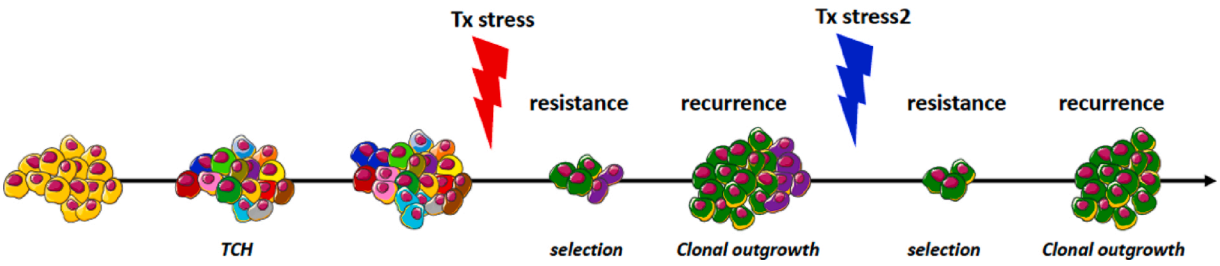

Fig. 1. Classic model of therapeutic resistance as the result of tumor cell heterogeneity. Resistance to therapeutic interventions has classically been attributed to genetic tumor cell heterogeneity: within the billions of cancer cells in a tumor, resistance to therapies evolves by random stochastic chance that endows at least one cancer cell with resistance to a particular therapy [4–24].

of genes provides an interesting yin-and-yang dynamic in regard to genomic mutation. It provides gene redundancy, allowing for mutation in one copy of a gene while retaining the wild-type information in other copies of the gene. This can provide genomic stability while also providing the opportunity for variation that can be inherited by subsequent generations [97]. Another advantage of polyploidy in Archaea is gene conversion, or the non-reciprocal transfer of information between homologous sequences of DNA [100–103]. Gene conversion in a polyploid cell allows the unicellular organism to avoid Muller's ratchet (the accumulation of deleterious genetic variation in the absence of recombination, as in asexual reproduction) [104–108]. One possible method to avoid the accumulation of deleterious mutations over time in haploid organisms such as Archaea is horizontal gene transfer, endowing a polyploid cell with the ability to construct and subsequently select advantageous genetic variants as a survival mechanism [100–102].

6. Bacteria

Bacteria have overlapping systems in response to diverse stresses including changes in temperature, nutrients, and toxins [98,109]. As in Archaea, increased cell size is associated with decreased predation and increased motility [98,110]. As a haploid organism with a single circular chromosome, bacteria too must escape Muller's ratchet to avoid the inevitable extinction that is associated with the random loss of fitness functions [111]. The main defence against the inexorable advancing click of the ratchet and erosion of the genome is recombination [112–114]. It has been demonstrated that in response to stress, a bacterium can increase its spontaneous mutation rate in the absence of DNA damage by upregulating the error-prone DNA polymerase Pol IV and down-regulating enzymes responsible for DNA mismatch repair (MMR) [109]. Multiple other mechanisms exist to increase mutability, including the movement of transposable elements. While these mechanisms result in increasing heritable mutation through generation of multiple mutants, evidence does exist supporting directed mutation. This type of "selected capture" of the beneficial mutation links the sensing of a useful genotype or phenotype to subsequent proliferation, leaving the new mutation unrepaired and immortalized [109,115–118]. This type of directed mutation could be considered a molecular Turing machine with oligonucleotides representing the "tape" and the restriction enzymes effecting transitions [119–122].

7. Eukaryota: protista

Protists are the earliest form of life that contain a nucleus and exist in haploid, diploid, and polyploid states [123–126]. The human pathogen *Entamoeba histolytica*, for example, routinely accumulates polyploid cells and reduplicate their genome several times before cell division occurs. Polyploidy may occur without nuclear division, and the checkpoints that normally prevent DNA reduplication until after cytokinesis in most eukaryotes are not observed in *E. Histolytica* [127]. *In vitro*, the fraction of polyploid cells increases with serum nutrient depletion and decreases again when fresh serum is resupplied [128]. The lack of nutrients, therefore, appears to delink of cytokinesis and karyokinesis from DNA replication [128].

Another protist group, the ciliates, demonstrate both transient and persistent polyploidy. In ciliates, the individual cell has two nuclei each with a specialized role. The "somatic" nucleus builds up extreme polyploidy (~1000 N) while the "germ" nucleus leads to daughter cells. Although diploid, the germ nucleus genome results from both gene loss and several events of WGD. The fitness increase through the persistent polyploidization events (WGDs) is not entirely clear, but it is argued that the increased gene dosage provides for increased metabolism [129].

Protists also appear to demonstrate gene editing. For instance, the ciliate germ nucleus may be fragmented by imprecise elimination (i.e., transposons or microsatellites), resulting in new DNA sequences that can be passed to progeny as heritable mutations [130,131]. Internal gene editing in the somatic nucleus (through mechanisms including DNA polymerase slippage, internal double strand breaks, and recombination reaction transposition), may increase fitness to changing environmental conditions during the lifetime of the organism and in the absence of cell division [132–134]. Upon cell division, the progeny would already possess heritable changes that improve their survivorship under the changed conditions. The haploid Foraminifera *Reticulomyxa filosa* utilizes polyploidy to escape Muller's ratchet [135]. The presence of multiple genomes permits lateral gene transfer, allowing the organism to generate mutations that can be tested for fitness while still maintaining a wild-type genome to ensure stability [98]. It remains unclear if this is an example of self genetic modification versus a form of heritable variability [136–138].

8. Eukaryota: fungi

The first WGD to be discovered in unicellular eukaryotes was in the yeast *Saccharomyces cerevisiae* [93,139]. Yeast cells that are polyploid

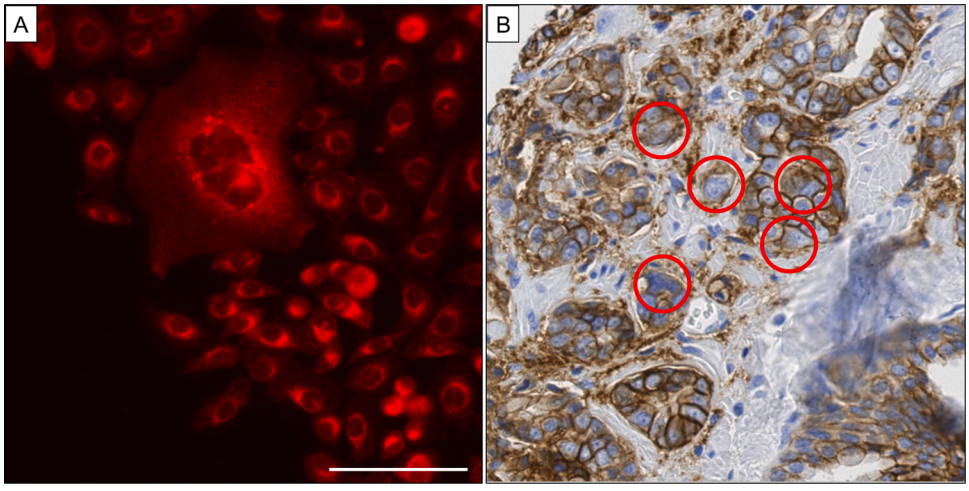

Fig. 2. Polyaneuploid cancer cells. PACCs are observed in cell culture: Panel A demonstrates untreated DU145 cells stained with Nile Red for contrast (scale bar = 200 um). PACCs are also observed in patients: Panel B demonstrates clinically localized prostate cancer (Gleason pattern 4 primary adenocarcinoma) stained with EPCAM to delineate cell borders, examples of PACCs indicated by red circles.

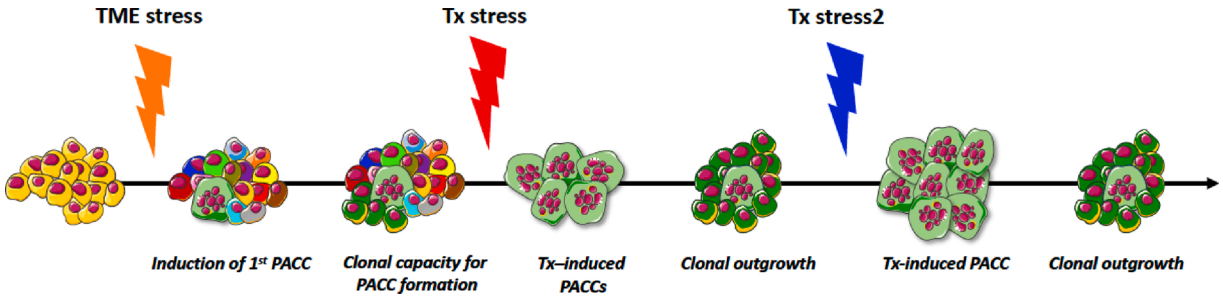

Fig. 3. Therapeutic resistance as the result of tumor cell heterogeneity allowing PACC formation. We hypothesize that resistance to therapeutic interventions is the result of access to an evolutionary/developmental polyploid program that increases DNA content, increases cell size, and induces quiescence as a result of environmental or therapeutic stress. The quiescent state allows the cancer cells to exit the cell cycle and avoid DNA damage and is a universal mechanism of therapeutic resistance that is mutation agnostic [26].

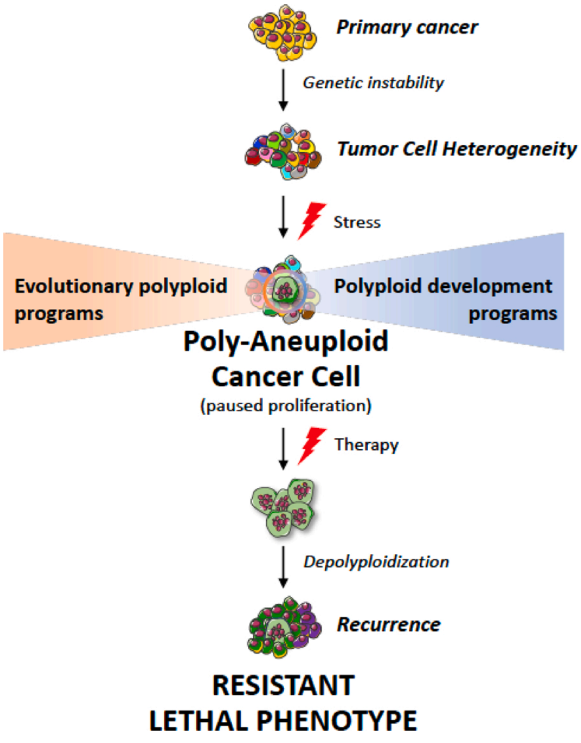

Fig. 4. Access to evolutionary and developmental programs that enable polyploidization and quiescence are the provide the key to understanding PACC structure and function. The formation of polyploid cells is by no means unusual in nature and is observed across both unicellular and multicellular eukaryotic organisms. Plants, fungi, and invertebrates, as well as vertebrate animals demonstrate polyploid cell formation during both development and temporal crises. Once PACCs are formed, they have the necessary characteristics to survive the catastrophic stress of therapy and survive to provide population rescue to a tumor [26].

and/or aneuploid survive better and evolve faster under changing and adverse conditions [140]. It has been inferred that this higher fitness is a result of their access to more beneficial mutations and therefore adaptability to novel settings [140–143]. In addition to polyaneuploid yeast cells accessing beneficial mutations or purging deleterious mutations, it has been suggested that cell size is mediated by dosage sensitive genes, i.e., genes that increase cell machinery, thereby increasing fitness [144–148]. Yeast form polyploids through meiosis without cytokinesis in response to toxins or adverse physical conditions. This permits rapid evolution of appropriate stress responses and later a return to a euploid state [140,148–150]. For example, recombination rates are many orders of magnitude higher in *Candida Albicans* during depolyploidization [151]. It is unclear if depolyploidization is by genetic variability in

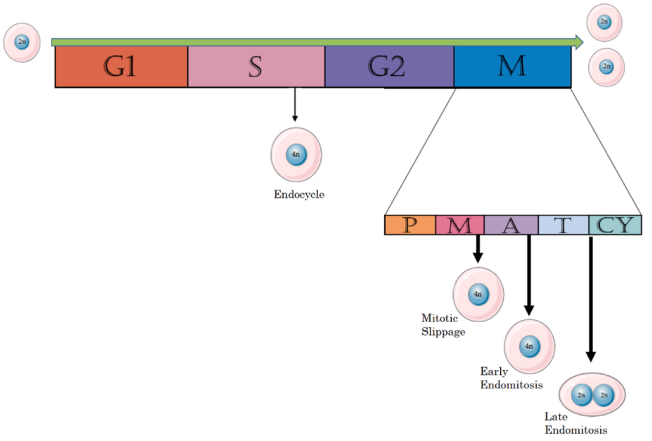

Fig. 5. Cellular mechanisms that generate polyploidy. In addition to cell fusion, polyploidy can be generated through endocycling, mitotic slippage, or endomitosis [71–77]. Endocycling also (endoreplication or endoreduplication) is the replication of DNA in S-phase without the cell entering mitosis. This results in a single 4 N nucleus. Mitotic slippage occurs when a cell exits the division cycle just prior to anaphase at the spindle assembly checkpoint, resulting in a single 4 N nucleus [71]. Endomitosis (cytokinesis failure) occurs during anaphase, resulting in a single 4 N nucleus, or in telophase, resulting in a multinucleated cell [40,76,78–80].

progeny with associated death of unfit offspring or accompanied by self genetic modification with the direct generation of only fit progeny.

9. Eukaryota: plantae

The main route for plant cells to increase volume is by modulating the cell cycle to engage in endocycling [152,153]. The resulting polyploid cells are either mononuclear (with or without separated chromatids) or multinucleated depending on when in M-phase the skip to G1 occurs. Such endoploidy can be somatic and present in only in specific cell types [154]. This differs from plants with species poly ploidy in which all of the somatic cells in their normal state possess a baseline level of polyploidy.

Somatic polyploidy contributes to plant development, function, and whole plant fitness [155,156]. The increased size of polyploid cells provides altered cellular functionality and organismal adaptability. Germinating seedlings utilize polyploidy to enlarge cells that accelerate the stem's emergence from the ground. Vacuoles in these cells provide filler that allows fewer cells to provide the same linear growth, and these vacuoles store energy for additional cell division [157]. Upon emergence from the ground, light negatively regulates endocycling and the mitotic cell cycle returns [158]. In adult plant roots and leaves, endoreplication can substitute for cell proliferation during harsh times such as

Table 1
Evolutionary Polyploidization Programs.

Organism taxa	Type of Program	Purported advantage	References
Archaea	- DNA replication without cell division	- Phosphate storage - Avoid predation - Increased motility - Increased gene dosage - Gene conversion	[94,95,96,97,98,99,100,101,102,103,104,105,106,107,108]
Bacteria	- DNA replication without cell division	- Enable quiescence - Increased mutation rate - Downregulation of error-correcting replication enzymes - Directed mutations	[109,110,111,112,113,114,115,116,117,118,119,120,121,122]
Protists	- Endomitosis	- Increase metabolism - Enable quiescence - Gene editing	[123,124,125,126,127,128,129,130,131,132,133,134,135,136,137,138]
Fungi	- Meiosis without cell division	- Increased gene dosage - Increased mutation rate	[139,140,141,142,143,144,145,146,147,148,149,150,151]
Plants	- Endocycling - Endomitosis	- Increased storage - Predator protection - Increased gene dosage	[152,153,154,155,156,157,158,159,160,161,162,163,164,165,166,167,168,169,170,171]
Drosophila	- Endocycling	- Increased metabolism	[172,173,174,175,176,177,178,179,180,181,182,183]

Table 2
Development Polyploidization Programs.

Cell type	Type of Program	Purported advantage	References
Trophoblasts	- Endocycling	- Placental development - Embryo nutrition	[189,190,191,192,193,194,195]
Keritinocytes	- Mitotic slippage	- Buffer for toxin stress - Increased genomic stability	[154,185,196,197,198,199,200]
Megakaryocytes	- Endomitosis	- "Efficient" platelet production	[201,202,203,204,205,206]
Macrophages and osteoclasts	- Cell fusion - Endomitosis	- Increased cell function	[207,208,209,210,211,212,213,214,215,216,217]
Myocytes	- Endocycling - Endomitosis	- Increased metabolism	[218,219,220,221,222,223,224,225,226,227,228]
Hepatocytes	- Endomitosis	- Increased genomic stability - Increased genetic diversity - Buffer for toxin stress - Increased metabolism	[229,230,231,232,233,234,235,236,237]

lack of water, sunlight, or nutrients [76]. For example, during episodes of drought, polyploidy compensates for cell loss by increasing leaf cell size thus moderating water loss [159].

The induction, direction, and termination of polyploidy in plants are not fully understood. Light and hormones can play a role [160]. For example, darkness or nutrient deficiency can induce endoploidy and light stops it [161]. Increased cell size is associated with a proportionally reduced access to and transport of metabolites into the cell as well as upscaling of organelles (e.g. ribosomes, mitochondria) [74,162–166]. Moreover, the increase in cell size and therefore distance for oxidants to diffuse can create an imbalance in reactive oxygen species (ROS) that triggers a shift in metabolism as well as delays the transition from prophase to prometaphase [167–171]. Thus, ROS can drive plant cell enlargement to facilitate chances for survival during stress. Access to programs for non-oxidative metabolism, inactivated apoptosis, and fitness in unknown future conditions would make polyploidy a beneficial strategy during harsh times.

10. Eukaryota: animalia (*Drosophila*)

Polyploidy has been extensively studied in *Drosophila* particularly in regard to development and wound healing [172–177]. Cell size and number are tightly regulated, resulting in an approximate total cell mass that changes in response to whole organism metabolic demands [176,177]. There is evidence in *Drosophila* showing that increased ploidy suppresses cell death in response to DNA damage. Polyploid cells may suppress the expression of pro-apoptotic genes [173,178]. Ploidy and cell size utilize Myc as a central regulator which influences multiple signaling pathways, including ribosome biogenesis as well as Dpp, Hpo, insulin, and mTOR [175]. During the larval stage, many of the fruit fly's cells stop dividing and undergo several cycles of endoreplication, reaching ploidies of >1000 N [179]. This increased DNA material amplifies a cell's biosynthetic capacity [179–181]. Tissue repair in drosophila requires the specialized functions of polyploid cells as well as the proliferation of diploid cells. This balance of polyploid and 2 N cells appears to be mediated by Myc expression [175,179,182]. Through regulation of CyclinE, Myc induces initiation of the endocycle, resulting in cells entering S phase but skipping mitosis [73,175,179,182,183]. Throughout, Myc provides a major driver of mitosis/endocycle coordination [78,89,90]. Myc globally amplifies transcription and decouples DNA synthesis and mitosis, resulting in polyploidy.

11. Development programs for polyploidy across human tissue

Noncancerous cells that form polyploids provide clues into the significance of increased size and increased DNA content of PACCs. PACCS may be able to epigenetically access cellular programs typically restricted to subsets of normal tissue cell types, e.g., megakaryocytes, keratinocytes, macrophages, osteoclasts, and trophoblasts as well as hepatocytes and myocytes [71,76,91,92,172,184–187]. A common theme across these tissue types is a need for the cells to amplify metabolic function to fulfill a physiologic need or enhance survivorship under stress. To generate muscle hypertrophy in response to exercise or injury stress, cardiac myocytes utilize polyploidization. Bone marrow macrophages fuse to create multinucleated giant osteoclasts allowing them to produce higher quantities of acid for dissolving bone matrix. In response to chronic infection, macrophages form giant multinucleated histocytes that can engulf foreign bodies.

In addition, polyploid cells are necessarily, at least transiently, in a state of proliferative cell cycle arrest, defined as G0 or quiescence. In multicellular organisms, the non-proliferative nature of terminally differentiated cells is essential for whole-organism tissue organization and fitness. Restricting proliferation of polyploid cells, especially in tissues experiencing stress and at risk for DNA damage, protects the cell lineage and, consequently, the organism as a whole. Quiescence, therefore, serves to isolate the effects of any DNA damage or disorganized inherited variation, preventing any deleterious (and possibly cancer-initiating) variation from being inherited by future generations of cells [188].

12. Trophoblasts

Placental trophoblasts form giant cells through endoreduplication,

resulting in high levels of ploidy. These large cells are associated with embryo implantation and placental development [189–191] assisting with placental connection and protection. The switch from the mitotic cycle to the non-proliferative endocycle has been extensively studied in trophoblasts and centers on a G2 decision point [192]. This decision point is regulated, in part, by the zinc finger transcription factor Snail which mediates expression of cyclins A and B. In addition, cyclin E coordinates the G1 to S phase transition and is essential for successful endocycling [192–194]. Simultaneously, the cyclin B/Cdk1 complex is not activated, resulting in inhibition of the mitotic cycle [193]. As demonstrated for yeast, trophoblasts undergo depolyploidization as the blastocyst forms [195].

13. Ectoderm: keratinocytes

Keratinocytes are constantly exposed to mutagens in the form of UV irradiation and environmental toxins [185]. Polyploidization may provide a mechanism to protect genome integrity by providing multiple copies of genes and restricting cell division. Some estimates place the percentage of polyploid cells in normal epidermis at up to 50 % [185]. In support of this idea, treatment with genotoxic agents induced differentiation and polyploidization in dividing keratinocytes [154,196]. This suggests that keratinocyte differentiation responds to DNA damage through mitosis checkpoints [197–200]. Similar to what is observed in drosophila, endoreplication appears to be stimulated by Myc and accumulation of cyclin E [154]. Keritinocytes, in response to DNA damage, progress through S-phase but arrest in G2/M which results in polyploidy through mitotic slippage via modulation of multiple checkpoint molecules, including depletion of Cdk1, Plk1, or AUR-A [196,198,198,199,200]. In addition to providing increased gene dosage, it has been suggested that polyploidy with concomitant increase in cell size may allow for cell survival and maintenance of barrier function [200].

14. Mesoderm hematopoietic lineage: megakaryocytes

Megakaryocytes are large (50–150 μm) differentiated cells dedicated to the production of platelets in mammals [201–204]. As these cells differentiate and mature, megakaryocytes undergo endomitosis, resulting in a polyploid nucleus that is 16 N on average, but has been observed up to 128 N [201–204]. As they increase in size, megakaryocytes exhibit an invaginated membrane system that is continuous with the plasma membrane, permeates the cytoplasm, and provides the extra membrane necessary for platelet formation and budding [205]. The large size may be necessary to have enough material to generate platelets. Furthermore, polyploidy may amplify the production of RNA and proteins for increased cellular metabolism and platelet production. The increased genomic material and increased cellular machinery results from the cell cycle stalling during late cytokinesis [72,79,204,206]. Telophase takes place with the formation of an apparently normal midzone and cleavage furrow. This is followed by rapid regression of the furrow resulting in a single cell with a single nucleus [201]. Molecular studies reveal that cell cycle disruption is mediated by a series of transcription factors (e.g, RUNX1, FLI1) that interfere with the RhoA pathway and regulate cyclins D and E [201].

15. Mesoderm hematopoietic lineage: macrophages and osteoclasts

Multinucleated macrophages have been observed in multiple granulomatous diseases such as tuberculosis, leprosy, and histoplasmosis, among others [43,207,208]. Multinucleated macrophages commonly form via cell-cell fusion through a well delineated series of steps that include pre-fusion priming of the cells, cell-cell adhesion, membrane fusion and multi-nucleation, and post-fusion reprogramming [209,210]. While it has been demonstrated in multiple systems that multinucleated macrophages can be formed by cell fusion in response to a variety of cytokines including IL4, proliferating macrophages in granulomas may also utilize aborted cytokinesis to increase in size and genome content [43,208,211,212]. The inflammatory microenvironment is a threat to DNA integrity due to the presence of ROS. In macrophages, if DNA double strand breaks are detected, the MRN complex (MRE11, NBS1, RAD50), is activated, leading to a robust DNA damage response (DDR). The DDR is activated by ATM kinase with subsequent downstream activation of CHK2 and p53 [208,213,214].

Bone marrow macrophages also fuse to form osteoclasts, large multinucleated cells for lysing bone matrix [43,209]. Polyploidy in this case serves to increase the production of cell products. Prior to fusion, pre-osteoclasts exit the cell cycle, presumably to protect their genome integrity [215–217]. Fusion is mediated by DC-STAMP, which in turn is regulated by multiple transcription factors, including c-Fos, NFATc1, PU.1, and NF-kb [43].

16. Mesoderm mesenchymal lineage: myocytes

After birth, cardiomyocytes halt cell division and increase their size in response to injury via WGD [187,218]. Polyploid cells with a single nucleus arise via endocycling and multinucleated cells arise through failed cytokinesis. This failed cytokinesis occurs late in the cell cycle during abscission, at which time the cytokinetic furrow regresses [187,219,220]. Entry into S-phase is mediated by the induction of cyclin D1 but M-phase entry is inhibited by the inactivation of CDK1 [221–223]. Rather than dedifferentiate to allow transit through the cell cycle with subsequent hyperplasia, cardiomyocytes increase in size and increase their number of contractile sarcomeres via hypertrophy. This suggests that the increased ploidy both protects the cell lineage from possible DNA damage and underlies a need for increased transcriptional output for subsequent protein synthesis and metabolism [187,219,220]. Polyploid vascular smooth muscle cells have been observed in chronically hypertensive animals [224]. As polyploid vascular smooth muscle cells increase in DNA content, they increase in size with a concomitant increase in RNA and protein. This increase is about twofold in tetraploid cells and fourfold in octaploids [176,225–227]. It appears that polyploid formation occurs in response to oxidative stress [228].

17. Endoderm: hepatocytes

In mammals, hepatocyte polyploidy contributes to both post-natal development and tissue regeneration throughout life. Both single and multi-nucleated polyploid cells can occur depending on the polyploidization pathway [71,184,229,230]. Polyploidy of hepatocytes results from endoreplication, or from failure to complete cytokinesis [229,231,232]. Though controversial, cell fusion seems to provide a rare but recurrent process for generating polyploid hepatocytes under physiological conditions [232]. Though the mechanisms for generating polyploidy in hepatocytes are known, the role played by these polyploid cells remains unclear. It has been demonstrated that proliferating hepatocytes produce a diverse population of progeny with multiple chromosome imbalances. It has been proposed that hepatocytes generate genetic diversity allowing them to adapt to xenobiotic or nutritional injury [71]. Alternatively, increased genomic material may provide a buffer against gene loss (i.e, providing a redundant genome) that would prevent cells from performing their whole organism function [71,233,234]. In addition, and not mutually exclusive to other postulated roles, polyploidization could provide increased cellular machinery as a buffer against oxidative stress [235–237]. A further potential reason for polyploidization may be the need to redirect energy to the production of cellular materials (e.g., RNA, proteins, or lipids) to maintain or increase metabolic activity when resources are limited such as during postnatal growth or regeneration after partial hepatectomy [91,235]. In support of this explanation, polyploid cells have few differentially expressed genes as compared to diploid cells. In these polyploids, WGD does not induce mutations or transcription reprogramming. Instead, they exhibit

increased transcriptional/translational production of cell materials [91].

18. Relevance of evolutionary and development polyploid programs to PACCs

Access to evolutionary and developmental programs that enable polyploidization provide the key to understanding PACC structure and function (Fig. 4). While the evolutionary programs emphasize the complementary response programs of genomic stability and heritable variation, the developmental programs emphasize response programs that allow survival and adaptation to stress secondary to metabolic needs. All of the development programs utilized by normal tissue cells provide roadmaps for understanding the mechanisms by which polyploidy is generated in PACCs. Megakaryocytes provide an extreme example in the human body of multi-lobulated single nuclei. They are a prime example of increasing DNA material to produce more cellular building blocks to increase the machinery necessary to produce platelets. If, for example, PACCs have a single multi-lobulated nucleus versus multiple nuclei, it is likely that they are accessing the development program utilized by megakaryocytes to endocycle. Determining the true nature of PACC nuclear content is a high priority.

Throughout evolution, it appears that polyploidization has been preserved across species as a rapid response to environmental stress by increasing cellular functional capacity and contributing to genome stability while halting proliferation, thereby protecting organisms from deleterious mutations [185,186,238]. The protists, for example, demonstrate that polyploidization increases cellular capacity for increased fitness rather than through increases in gene dosage. Although the protists appear to have a versatile capacity for gene-editing during their polyploid phases, the link between gene editing and heritability remains unclear. Polyploid yeast cells, for example, have been shown to have higher fitness due to initial dampening of deleterious mutations and subsequent access to beneficial mutations that lead to increased heritable variation [140–143].

The depolyploidization programs described in fungi represent early evolutionary programs that form the basis for meiosis programs in eukaryotes and subsequently the presumed depolyploidization programs of PACCs [62,239–241]. For example, within a population of cancer cells induced by radiation to undergo mitotic catastrophe, a subset of PACCs are formed which subsequently undergo depolyploidization to form non-polyploid progeny [62,242]. It appears that successful depolyploidization is linked to expression of meiotic-specific pathway genes including SYCP2, SYCP3, DMC1, SPO11, REC8, STAG3, and MOS [62]. Alternatively, in ovarian cancer, PACCs appear to form in response to stress and produce progeny by amitotic mechanisms, e.g., budding [38,243,244]. It has been demonstrated that a single multinucleated cancer cell can reform an entire tumor population [55]. The mechanisms by which PACCs produce progeny need to be delineated in detail.

19. Potential for PACCs to create therapeutic resistance mechanisms

The generation of WGD of aneuploid cancer cells and the resultant polyaneuploidy is now well documented in the cancer literature [236,245]. It is not established, however, whether this polyploidization is 1) an obligate step of a resistance program of randomly generated clones as part of existing tumor cell heterogeneity [4,10–12,15]; 2) a means of inducing quiescence to increase cellular machinery to survive while protecting genomic material for future progeny [30,38,42,45,57,246,247]; 3) a means to increase genetic stability to prevent apoptosis in a cell with damaged DNA while also allowing increased genetic instability to create heritable variation [75,76,92,173,248–251]; and / or 4) a potential means of generating self genetic modification (Fig. 6).

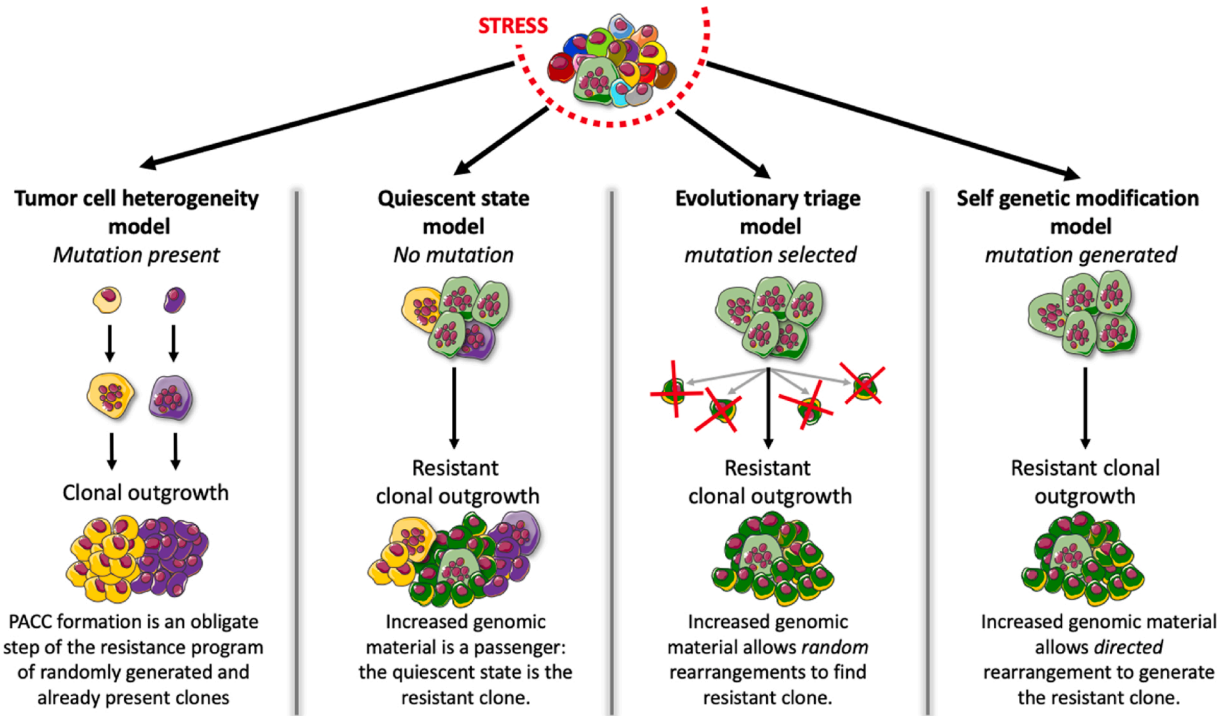

Fig. 6. Models to explain how PACCs may contribute to the evolution of resistance. Polyaneuploid cells appear to be part of a central pathway in the generation of therapeutic resistance. It is not established, however, whether this polyploidization is 1) an obligate step of a resistance program of randomly generated clones as part of existing tumor cell heterogeneity [4,10–12,15]; 2) a means of inducing quiescence and increasing cellular machinery to survive while protecting genomic material for future progeny [30,38,42,45,57,246,247]; 3) a means to increase genetic instability to create heritable variation [75,76,92,173,248–251]; and / or 4) a means of generating self genetic modification [109,115–118].

19.1. The ecological concept of a fundamental niche

Polyploidy either protects the cell through quiescence, potentially accelerated evolution, or both. Accelerated evolution would occur through two pathways, either evolutionary triage through heritable variation and, controversially, some form of self genetic modification, also referred to as genetic assimilation, gene editing, or adaptive mutation. Regardless of the pathway, a clear definition of the ecological concept of a fundamental niche is required [252].

The fundamental niche of an organism – or of a cancer cell – represents the range of environmental conditions over which the organism can maintain a viable population. For cancer cells, the fundamental niche is the tumor microenvironment that allows the cells of the newly initiated clade to proliferate and accumulate mutations that lead to the establishment of a successful tumor population. Experiencing conditions inside or outside of its fundamental niche pose two very different eco-evolutionary challenges. Evolution acting on organisms within their fundamental niche selects for adaptations that allow the organism to succeed relative to others. While individuals will increase in frequency, those with a less fit trait will die off due to the presence of the fitter one. This is the typical context for evolutionary triage where far more individuals are born than can survive. Having more heritable variation accelerates evolution by increasing the likelihood of having fitter individuals causing the less fit to die off. It is well accepted that heritable variation is the predominant driver of natural selection for organisms primarily experiencing ecological conditions within their fundamental niche (Fig. 7).

What happens when environmental conditions deteriorate so drastically that the organism it is now outside of its fundamental niche? In the absence of a rapid return to favorable conditions, the organism will migrate to more favorable conditions or simply go extinct at that location. Alternatively, the organism could enter a protective quiescent state, halting reproduction and therefore protecting its genomic material from assault, and wait until conditions or its own fitness change. As another strategy, the organism could evolve traits that expand its fundamental niche to include the otherwise harsh conditions. In the tumor cell heterogeneity model of therapeutic resistance, a resistant clone has randomly been generated in the population through stochastic mutations as a result of genetic instability (Fig. 6). In this model, polyploidization represents a transitory phenotype that the already-resistant clones employ prior to repopulating the tumor. This transitory cell state most likely represents a strategy of the cell to enter quiescence to ensure genome integrity in the presence of the therapeutic stress.

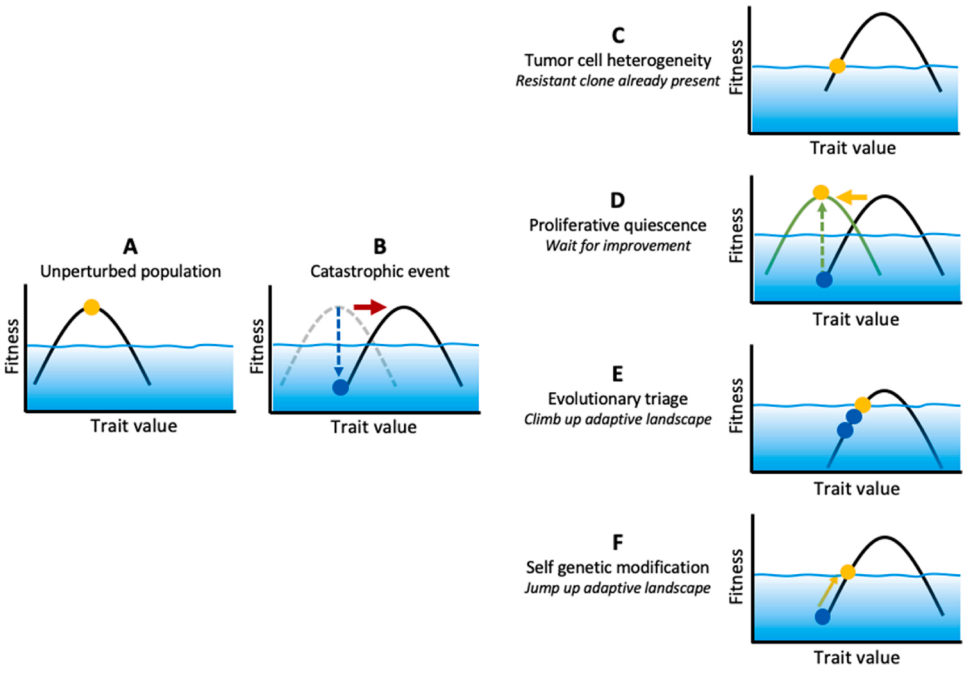

Fig. 7. Evolutionary and ecological dynamics along adaptive landscapes. Panel A shows the adaptive landscape (solid black line) for a population within its fundamental niche (shown as the solid yellow circle) that demonstrates both ecological fitness and evolutionary equilibrium (peak of the adaptive landscape) where the y-axis is fitness (measured as per capita growth rate) and the x-axis represents a heritable trait value. **Panel B** demonstrates a drastic change to the environment that dramatically shifts the original adaptive landscape (dotted line) to a new adaptive landscape (solid line). Under these new conditions the population with the identical trait value (solid blue circle) may find itself outside of its fundamental niche (i.e., "underwater") and therefore nonviable. **Panel C**: Alternatively, consistent with the tumor cell heterogeneity model, this catastrophic loss in population size may identify a rare clone that can survive in the altered landscape and recover its viability (yellow circle at the "waterline"). **Panel D:** If the population can wait out the change in landscape (i.e., "hold its breath"), the population can be rescued if conditions improve and the original adaptive landscape is re-established. **Panel E**: Evolutionary triage can drive the population's trait value along the new fitness landscape until an eco-evolutionary equilibrium is re-established. While evolutionary triage is effective to adapt to slow changes in the landscape, it is unlikely to be effective in response to a catastrophic event. **Panel F**: If the population's trait value is outside of its fundamental niche (solid blue circle) then it is doomed to extinction unless it can evolve fast enough to achieve a viable trait value (solid yellow circle). Via PACCs, the controversial concept of self genetic modification may provide the most efficient (or perhaps the only) way for this evolutionary jump to occur. This will expand the fundamental niche of the population to now include the otherwise disastrous conditions. Once recovered (solid yellow circle), evolutionary triage can resume evolution towards an eco-evolutionary equilibrium.

19.2. Evolutionary triage to create heritable variation

Natural selection by evolutionary triage is particularly effective when the cancer cell population is well within its fundamental niche [253–256]. Within its fundamental niche, the tumor environment is favourable and there are no stressors that threaten the viability of the population of cancer cells. Cancer cells can persist even if their total phenotypic trait values are far away from an evolutionary optimum and progressive evolution is underway towards ever more fit individuals. Under these circumstances, through proliferation and cell deaths, natural selection can drive cell lineages towards evolutionary optimum. The only threat to the extant cancer cells is their replacement by cells with fitter trait values. When a species or cancer cell population resides in its fundamental niche, evolution is more about outcompeting a neighbour rather than surviving exogenous environmental stressors.

Theoretically, PACCs could play an evolutionary role by accelerating evolution by generating a wider array of heritable variation in their offspring. These 2 N + offspring would then undergo evolutionary triage as fitter variants prosper at the expense of other 2 N + members of the population. When the cancer cell population resides within its fundamental niche, PACCs would allow for faster evolutionary tracking of changing tumor microenvironments, faster evolution towards evolutionary optimum, and more rapid diversification of cancer cell types within a heterogeneous tumor. A mechanism by which PACCs simply increase the heritable variation among their 2 N + offspring would be an inefficient way of generating evolutionary rescue when the stressor pushes the cancer cell population outside of its fundamental niche. By creating genetic shotgun blasts of variation, PACCs could create what Goldschmidt termed “the hopeful monster” [257–259]. For cancer, the hopeful monster would be that rare chance 2 N + cancer cell that now possesses traits that make the stressful environment a part of its fundamental niche. Such evolution is seen as being saltatorial, creating large jumps in the trait values of the organism. While controversial, such evolution does occur in multicellular animals and is also associated with whole species polyploidy and a source of evolutionary rescue [260–263].

If genetic instability followed by evolutionary triage were the primary mechanism at play, multiple different clones, each with unique genetic variation, would be produced, but only a subset of these would survive while the rest would die off. When studied in an *in vitro* microfluidics environment designed to simulate stress, PACCs generate resistant progeny without concomitant generation of multiple non-viable progeny [51,68]. This suggests an alternative mechanism other than genetic instability (Fig. 7).

19.3. Quiescence as a mechanism to protect the genome

The polyploid program, as an inherent non-proliferative quiescent state, protects cells from immediate genome damage. In this cell state, PACCs can adapt a new function for WGD that is not a traditional evolutionary or developmental reason for polyploidization. Ecologically, the PACC state may provide higher survivorship during times of extreme stress. Under the stressor, a population comprised only of 2 N + cells far outside of its fundamental niche may have extremely low viability. Even as the population collapses, PACCs may provide a means for the cancer cells to simply survive [51,68,264]. Once conditions improve, the PACCs can re-establish the highly proliferative 2 N + state. In this model, there is no mutation to generate a resistance phenotype - quiescence to protect genome integrity is sufficient. This is akin to many protists forming an encysted non-proliferative state that survives harsh environmental conditions that are unfavourable for proliferation [128]. Quiescence, senescence, polyploidy, and cancer are clearly closely related but their intersecting biology remains poorly understood [45]. As previously noted, these large amorphous cancer cells were disregarded and not deemed to be functionally important because they were considered to be irreversibly senescent or destined for mitotic catastrophe. Our own studies suggest that only a minority of PACCs induced by chemotherapy exhibit canonical senescence biomarkers (data not shown). Studies conducted in yeast, Drosophila, cancer models, and clinical data suggest, however, that the polyploid state mediates therapy-resistant phenotypes [46]. Furthermore, there is evidence that a population of cancer cells can survive chemotherapy and radiotherapy by entering a reversible senescent state called therapy-induced senescence (TIS) that displays many of the features of the normal physiological senescence phenotype [46,265]. Determining the relationship of stress induced whole genome doubling with concomitant exiting of the active cell cycle with be critical in defining the roles of quiescence versus senescence versus paused proliferation in PACC biology.

19.4. Self genetic modification as a mechanism to alter the genome

Effecting evolutionary rescue by producing a plethora of genetic variants would be wasteful of offspring as most would simply die and, at best, only a small percentage might have trait values that include the now stressful conditions as part of their fundamental niche. It would be much more efficient if PACCs used their capacity for increased RNA and protein production and increased intracellular genetic variability to assess their lack of viability under the stressor, and respond by enacting a metabolic solution that can then be back-encoded into the DNA itself, i.e., self-captured mutation [109,115–117]. This could then form the basis for budding off 2 N + cells that already carry a heritable solution to the stressor. Such evolution is controversial and has variously been described as genetic assimilation, gene editing, or adaptive mutations and is not widely accepted as an evolutionary mechanism [116,266–269]. In 1953, the founder of systems biology Waddington proposed genetic assimilation as a feedback between acquired traits and genetic encoding [270,271]. More recently genetic assimilation has been proposed for similar observations in yeast [272]. Bacteria have evolved overlapping systems to respond to a variety of stresses including changes in temperature, nutrients, and toxins [98,109]. Multiple mechanisms to increase mutability exist, including the movement of transposable elements, exist. While these mechanisms result in increasing heritable mutation through generation of multiple mutants, it has been argued that evidence also supports the selective capture of directed mutations [109,266–268,273–279].

Experimentally, we do not observe the death of mutant clones that should be generated by heritable variation. The acquisition of drug resistance appears to occur without the random mutations and creative-destruction explained by heritable variation [68,280,281]. Thus, it appears that PACCs have the means to assess the stressor, identify a solution, and then alter genes or epigenetics to respond appropriately. If this is true, rather than just being the recipe of inheritance dictating phenotypes and traits, the genetic architecture itself becomes a trait that offers a remarkable source of phenotypic plasticity that enables a form of self genetic modification. The capacity for self genetic modification would then be an adaptation produced by natural selection, providing an alternative and more efficient, albeit controversial, means for adapting to novel stressors [282,283]. Evolutionary triage may be too slow and too wasteful of unviable offspring to effect evolutionary rescue, particularly when conditions leave the current population far outside of its fundamental niche. Self genetic modification, if it exists, may be the pathway by which polyploidy allows single celled organisms and cancer cells to extend their fundamental niche to include otherwise disastrous conditions.

19.5. Genome chaos as a mechanism to alter the genome

These potential strategies to evolve the genome of PACCs in response to stress, regardless of mechanism, can be framed as a microevolutionary modifications - limited changes to the genome over time to improve survival. It is also possible that PACCs utilize a more macroevolution

strategy - large and rapid changes to the genome through the formation of chaotic genomes, e.g., through chromosome fragmentation [243,284–286]. Stress can lead to shattered chromosomes that can be randomly rejoined throughout the genome, resulting in chromothripsis and increasing karyotype complexity [284,287,288]. The genome system theory proposes that chromosomes act as gene topologic organizers (karyotype coding) and function to drive macroevolution through genome based rather than gene-based inheritance [284]. Understanding the interplay of microevolution and macroevolution systems will be critical to understanding how PACCs evolve resistance to microenvironment and treatment stresses [243].

20. Conclusion

The capacity for cells to assess stressors, identify solutions, and alter its genes or epigenetics in ways that results in viable solutions must have been advantageous since life inhabited settings where drastic environmental changes occur. Unicellular life has been present on Earth for most of its 4.5 billion year old history, and present for at least half of this time in surface environments where conditions like redox, light, and pH change ceaselessly [289–292]. Thus, organisms with capacities for assessing being removed from their fundamental niche, for enduring the time outside of the fundamental niche, and for accelerated evolution to yet again become viable, have been selected for over billions of years. Although not widely discussed in the cancer literature, polyploidy as a means to endure, come through, and maybe even assess the harsh conditions is likely a robustly evolved capacity for the many different kinds of cells and organisms that employ it during their life in variable settings.

PACCs represent a formidable challenge to managing and curing cancer. As the source of therapeutic resistance, PACCs appear to be the primary source of cancer's lethality. Targeting PACCs is a difficult task since they represent such a tiny fraction of the overall tumor cell burden and are actually formed in response to external stress, including therapy. Further work is required to understand the mechanisms by which PACCs are formed and how these mechanisms can be targeted. It is clear that multiple strategies will need to be combined that both eliminate the bulk a large number of the 2 N + cancer cells that make up the bulk of the cancer cell population and a strategy to kill the few in number, but critically important PACCs. One potential strategy will be to identify and eliminate or prevent the stresses that cause PACCs to initially form in the primary tumor microenvironment. This would prevent the initiation of lethal phenotype and formation of metastasis initiating cells. This opens the door for potential prevention strategies, potentially by inhibition of the formation of reactive oxygen species [293–295]. This type of strategy may not be possible, however, when an extrinsic stress such as chemotherapy is needed to eliminate large populations of proliferating cancer cells. Drawing from ecology, successful strategies will likely require an evolutionary double bind whereby an organism is forced to adopt an adaptive response to an environmental stressor which then makes it vulnerable to a second, different stressor [24,296].

For cancer populations, we envision that the first strike would take the form of an anti-proliferative agent, e.g., chemotherapy to eliminate the majority of 2 N + dividing cancer cells. Killing the surviving PACCs will require a novel second-strike therapy that specifically targets their unique vulnerabilities (Fig. 8). Clues to these potential susceptibilities are beginning to be identified. As the programs that control polyploidization are defined (Fig. 4), multiple inhibitors of the cell cycle checkpoints are clinically available, ready to be applied in strategic manners [297–301]. Aneuploidy itself and the necessity for appropriate chromosome segregation likewise offers multiple therapeutic targets [302–305]. Many agents along these pathways have been developed but have failed in the clinic because they have been given non-discriminately to the whole population of cancer cells. The large size and increased cellular contents of the PACCs offer potential targets secondary to altered metabolic pathways, e.g., lipid biosynthesis, as well as protein homeostasis and cell energetics [293,306,307]. Quiescence and therapy induced senescence also offer unique strategies, again if applied in a double bind fashion [308–310]. Moreover, the abnormal morphology of PACCs suggests that there may be unique cell surface antigen profiles that can be exploited for directed antigen-conjugation to deliver a toxic payload to the cells. Targeting PACCs must be a high priority as they are the key to therapeutic resistance and the incurability of cancer.

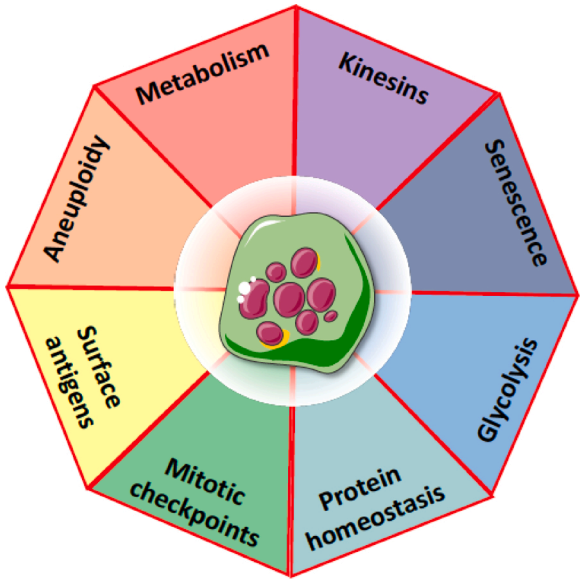

Fig. 8. Targeting polyaneuploid cancer cells for therapeutic destruction. Targeting PACCs is a difficult task since they represent such a tiny fraction of the overall tumor cell burden and are formed in response to external stress, including therapy. Drawing from ecology, successful strategies will likely require an evolutionary double bind whereby an organism is forced to adopt an adaptive response to an environmental stressor which then makes it vulnerable to a second, different stressor [24,296]. For cancer populations, we envision that the first strike would take the form of an anti-proliferative agent, e.g., chemotherapy to eliminate the majority of 2 N + dividing cancer cells. Killing the surviving PACCs will require a novel second-strike therapy that specifically targets their unique vulnerabilities. Vulnerabilities are being identified based on the unique structure and functions of the PACCs, including targeting the cell cycle [297–301], aneuploidy [302–305], metabolism [293,306,307], and senescence [308–310].

Declaration of Competing Interest

Dr. Pienta is a consultant for CUE Biopharma, Inc., is a founder and holds equity interest in Keystone Biopharma, Inc., and receives research support from Progenics, Inc. The other authors declare no conflicts of interest.

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ROS-induced cell cycle arrest as a mechanism of resistance in polyaneuploid cancer cells (PACCs)

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ABSTRACT

Cancer is responsible for the deaths of millions of people worldwide each year. Once metastasized, the disease is incurable and shows resistance to all anti-cancer therapies. The already-elevated level of reactive oxygen species (ROS) in cancer cells is further increased by therapies. The oxidative stress activates the DNA damage response (DDR) and the stressed cancer cell moves towards cell cycle arrest. Once arrested, the majority of cancer cells will undergo programmed cell death in the form of apoptosis. If the cancer cell is able to exit the cell cycle prior to cell division and enter a protected G0 state, it is able to withstand and survive therapy as a polyaneuploid cancer cell (PACC) and eventually seed resistant tumor growth.

Introduction

Globally, 9.5 million people died as a result of cancer in 2018 (Ferlay et al., 2018). In 2020, it is estimated that 1.8 million people across the United States will be newly diagnosed with cancer and of all diagnosed patients, 600,000 will die as a result of the disease (Siegel et al., 2020). Metastatic disease is responsible for 90% of all cancer related deaths (Pienta et al., 1557). Cancers confined to the primary site are considered curable and can be removed through primary therapies such as surgery or radiation. Once cancer metastasizes and spreads to sites across the body it is considered incurable (Pienta et al., 1557).

Metastatic disease is treated with systemically-administered anti-cancer therapeutics, such as chemotherapy, with the goal of killing the rapidly dividing cancer cells. Eventually, however, cancer becomes resistant to all types of anti-cancer therapies. While reducing total tumor burden, therapeutic exposure may also induce the emergence of a small subset of multi-therapy-resistant cancer cells that survive and seed recurrence following therapeutic intervention (Pienta et al., 2020; Norouzi et al., 2018). These cells show resistance not only to the applied therapy, but also other anti-cancer drugs and therapies applied following initial exposure (Pienta et al., 2020; Eccles and Welch, 2007). These resistant cells are present in small numbers in the primary tumor, having already evolved to the same lethal phenotype: resistance to external stress. It is hypothesized that they play a key role in the metastasizing potential of cancer and have been shown to activate as a response to stress, including chemotherapeutic insult (Pienta et al., 1557; Pienta et al., 2020).

The emergence – and survival – of these rare resistance-mediating cells may be attributed to a relationship between the cancer cell's stress response pathways and reactive oxygen species (ROS). When applied, anti-cancer therapies affect the internal homeostasis of the cell by damaging nuclear content and generating ROS (Perillo et al., 2020; Lord and Ashworth, 2012). The generation of ROS across the cell shifts the redox balance towards a state of oxidative stress (Perillo et al., 2020; Trachootham et al., 2008). Genomic instability is a Hallmark of cancer, and, therefore, various pathways of the DNA damage response (DDR) are already activated even in untreated and non-stressed cancer cells. The DDR can be affected by a number of factors ranging from the cell's cell cycle status to the type of DNA strand breakage (Davalli et al., 2018; Bartek, 2011). The DDR is not a single pathway, but instead is a suite of biochemical pathways and responses that sense DNA damage and determine the fate of the cell. Fates include repair during various stages of proliferation, slow cell cycling, and cell cycle arrest to allow for more extensive DNA repairs, or apoptosis in the case of

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damage extending past the point of repair (Davalli et al., 2018; Min and Spencer, 2019). Both therapeutic DNA damage and high ROS inflict their own damage on the nuclear contents of the cell, while regulating the DDR, apoptosis, and the cell's entry into cell cycle arrest (Perillo et al., 2020; Srinivas et al., 2019).

1.1. Reactive oxygen species as a moderator of cancer cell death

ROS are primarily produced as natural byproducts of cellular respiration. The generation and removal of ROS determines the cellular redox balance, contributing to the overall homeostasis of the cell (Trachootham et al., 2008; Ray et al., 2012). In general, all cells rely on the transcription of antioxidants, such as glutathione, to reduce ROS when levels increase past signaling thresholds. If ROS generation outpaces the reduction capabilities of the cell's antioxidants, the cell will experience oxidative stress and the mitochondria and nucleus will be damaged (Perillo et al., 2020; Ray et al., 2012). ROS molecules such as superoxide (O^{-}_{2}), hydroxyl radicals (OH^{-}), and hydrogen peroxide (H_2O_2) are present in the cytosol and organelles of all cells (Ray et al., 2012). Tightly-regulated levels of ROS play a critical role in both normal and cancer cells as initiators and regulators of various cellular signaling pathways (Trachootham et al., 2008; Ray et al., 2012; Zhou et al., 1521). In cancer cells, it is well documented that both ROS and antioxidant levels are increased compared to normal cell populations (Fig. 1A). (Noh et al., 2015; Kumari et al., 2018) The elevated, yet balanced, ROS levels can activate signaling pathways that contribute to cancer's ability to metastasize (Perillo et al., 2020; Zhou et al., 1521; Liao et al., 2019; Szatrowski and Nathan; Schieber and Chandel, 2014). For example, ROS promotes metastasis by aiding in EMT transformation pathways via TGF-β1 (Liao et al., 2019).

Anti-cancer therapy increases internal ROS levels by introducing exogenous agents to the cell, as well as, by causing extensive DNA damage thus causing a response of ROS generation (Perillo et al., 2020; Rowe et al., 2008). This causes the redox balance to shift in favor of oxidative stress, subsequently damaging organelles and altering signaling pathways (Fig. 1B). One damaging effect occurs in the nucleus, where DNA damage continues to accumulate from both therapeutic effects, as well as, the ROS-mediated oxidation of bases (Fig. 1B). (Perillo et al., 2020; Lord and Ashworth, 2012) The increase in oxidative stress also hinders the corrective capabilities of the cell's DDR pathways, inhibiting some, if not all, repair pathways (Fig. 1B). (Srinivas et al., 2019; Curtin, 2012) The DDR, unable to repair the DNA breaks, will signal the cell to move into cell cycle arrest to prevent the replication of damaged DNA. There are several possible outcomes following cell cycle arrest. If unable to repair its severely damaged DNA, the DDR can induce apoptosis, a form of programmed cell death (Fuchs and Steller, 2015). In a distinct mechanism, the elevated ROS levels across some cancer cells can also trigger programmed cell death through ROS-induced apoptosis (Srinivas et al., 2019; Liou and Storz, 2010). As a result of oxidative stress, pores along the mitochondrial membrane can be oxidized or the mitochondrial membrane may depolarize, allowing cytochrome *c* to be released into the cytoplasm, initiating the apoptotic cascade (Davalli et al., 2018; Srinivas et al., 2019; Liou and Storz, 2010; Simon et al., 2000). Cancer cells can avoid apoptotic cell death by promoting the expression of anti-apoptotic proteins such as Bcl-2. It has been noted that an increase in Bcl-2 may also play a role in promoting antioxidant recruitment and generation during oxidative stress to manage ROS and mitochondrial membrane depolarization, thus evading apoptosis (Chong et al., 2014). It has also been reported that cancer cells are capable of re-working and optimizing pathways responsible for the re-generation of glutathione and other antioxidants (Noh et al., 2015). In doing so, the antioxidants are capable of reducing the oxidized membrane of the mitochondria and can help prevent the escape of cytochrome *c* (Assi, 2017). The mitochondrial membrane stabilization by antioxidants as a response to prolonged exposure of ROS can prevent the cancer cell from succumbing to therapeutic treatments (Liou and Storz, 2010); however, the hindered DDR still prevents the cancer cell from returning to the cell cycle (Davalli et al., 2018). The cancer cell remains in a steady state of cell cycle arrest.

1.2. Protective cell-cycle arrest

Exit from the cell cycle is a well-documented phenomenon in many cell types and organisms ranging from plants and yeast to animals and humans (Fig. 2A). (Valcourt et al., 2012) Various stimuli will induce a cell to exit the cell cycle and enter a “dormant” G0 state, including overcrowding and contact inhibition (Sun, 2014), absence of growth factors or nutrients (Zetterberg A Fau - Larsson and Larsson), and DNA or cellular damage (Barr et al., 2041). Though cell cycle exit is a survival strategy observed in many cancer types (Chang et al., 1999), often as a result of DNA damage from

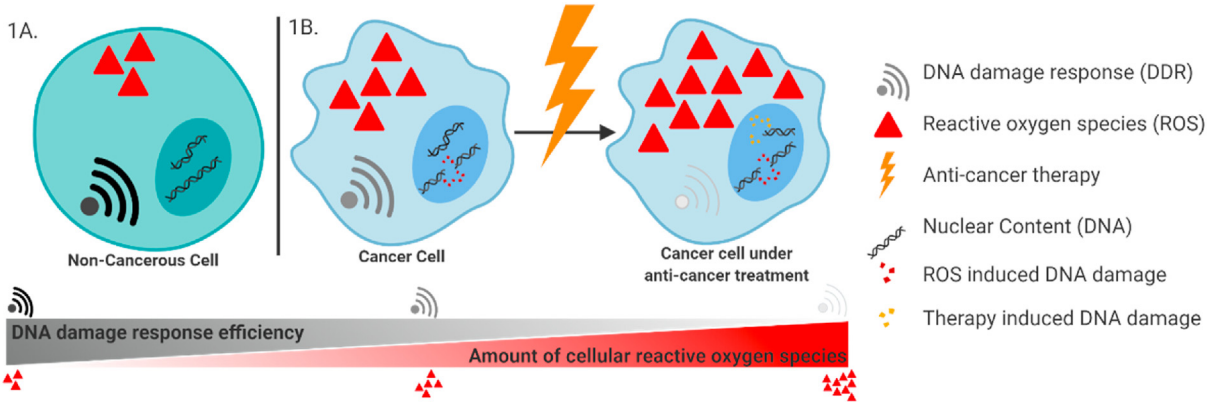

Fig. 1. A) In non-cancerous cells, reactive oxygen species (ROS) exist at moderate to low levels to aid in the regulation of various cell signaling pathways. The DNA damage response (DDR) is capable of repairing oxidative stress-induced DNA damage and the integrity of the genome is maintained. B) Cancer cells have an increased amount of ROS, shifting the redox balance of the cell. The increased oxidative stress inflicts damage on the DNA of the cancer cell, contributing to cancer's genomic instability. The DDR pathways, while activated from the ROS-induced DNA damage, are also hindered by the increased oxidative stress levels across the cancer cell. When anti-cancer therapy is applied to the cancer cell, the levels of ROS increase as a stress response. Both the ROS-induced and therapy-induced DNA damage activate the DDR; however, the DDR pathways are still hindered by oxidative stress and are not capable of repairing DNA at the rate it is being damaged.

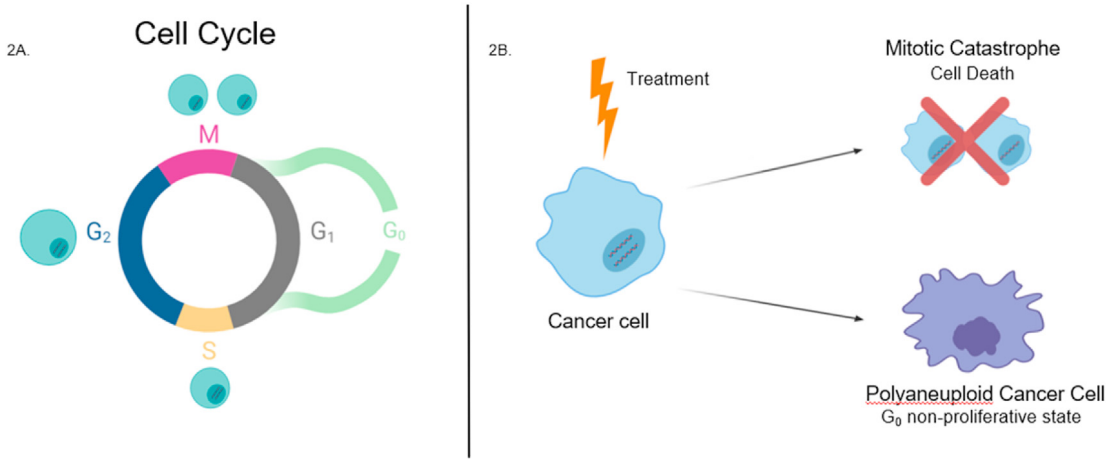

Fig. 2. A) In a normal cell cycle, the cell will go through various stages of replication and growth in order to divide. In G_1 (Growth 1), the cell will synthesize proteins and factors needed throughout the rest of the cycle, the cell will also begin to grow slightly in size. In S phase all nuclear DNA will be replicated, giving the cell two sets of genomic DNA. In G_2 (Growth 2) the cell will continue to grow in size and produce new proteins that it will need in the division process. Ending in Mitosis, the cell will finally begin the process of division, splitting apart its DNA and organelles to give rise to two identical daughter cells, who continue the cycle back into G_1. There is also a G_0 phase outside of the traditional cycle, used as a protective non-proliferative state that the cell can use to exit the cycle to repair DNA damage, when necessary. B) A cancer treated with therapy has two possible fates: 1) attempt to undergo cell divide and apoptose due to mitotic catastrophe, or 2) escape the cell cycle and not complete mitosis or cytokinesis and enter the protective G0 state as a PACC.

systemic anti-cancer therapies as chemotherapies (Roninson et al., 1368).

The goal of systemic therapy is to kill cancer cells, and in particular, the rapidly proliferating cells of a growing tumor. This is achieved by inducing an overwhelming amount of DNA damage (e.g., DNA poison cisplatin and DNA polymerase II inhibitor Etoposide) or by inhibiting some aspect of normal cell cycle (e.g., microtubule stabilizer docetaxel) to force the cell into apoptosis upon cell division (Barr et al., 2041; Lowe and Lin, 2000). In cancer, this DNA damage is compounded by an increase of damaging ROS. The addition of imbalanced ROS and cancer therapy-induced DNA damage overwhelms the DDR pathways within the cell (Srinivas et al., 2019) leading to either cell death via mitotic catastrophe or a complete exit from the cell cycle (Chen, 2016). When such excessive damage occurs, the cell must exit the cycle to repair the damage, tolerate it, or succumb to it via apoptosis (Shen et al.,

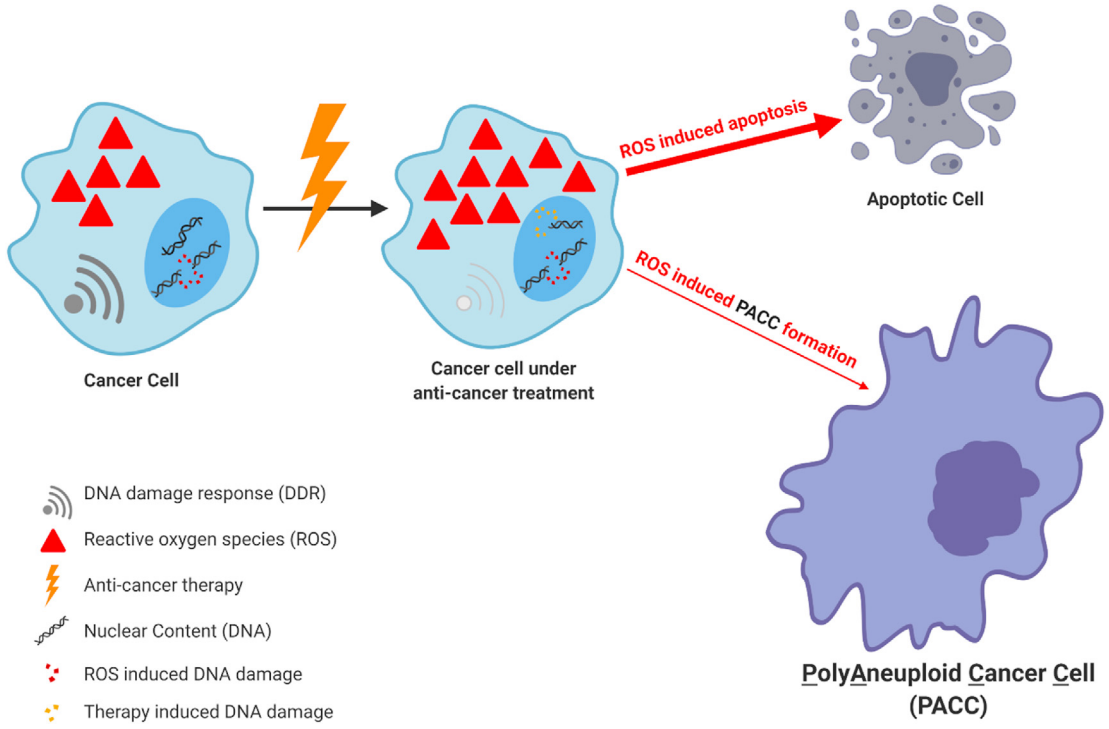

Fig. 3. In response to anti-cancer therapy and the subsequent surge in ROS, the cancer cell is limited in its survival options. The majority of cells will be overwhelmed by the increase in nuclear damage and oxidative stress, inducing cellular death. A smaller subset of cancer cells will prematurely exit mitosis, escape the cell cycle, and enter a protective, G0 state. It is in this state that the cancer cells are protected from anti-cancer therapy and can form into polyaneuploid cancer cells (PACCs). Once a break in therapy occurs, these PACCs can re-emerge equipped with non-selective resistance to all anti-cancer therapies.

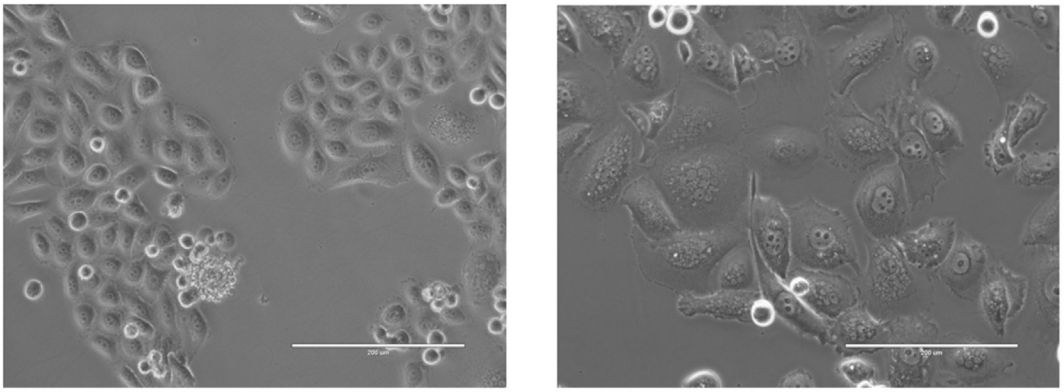

Fig. 4. Phase contrast imaging of an untreated control PC3 population and a PC3 population treated with 6 μM cisplatin 72 h following plating and treatment. (scale = 200 μm) PACCs are visibly apparent after treatment. **(Figure should be printed in color. 2 column fit.)**

2013).

To enter a protected non-proliferative state the cell must complete or abort its current cycle without triggering the cell cycle checkpoints that induce apoptosis (Barr et al., 2041). This will lead to the cell "slipping" out of mitosis to avoid potential mitotic catastrophe (known as mitotic slippage, early or late endomitosis, or acytokinesis) (Brito and Rieder, 2006), or bypassing mitosis altogether through endocycling (Erenpreisa et al., 1065), likely dependent on where in the cycle the DNA damage occurred. If a cell survives treatment-induced apoptosis by not completing cytokinesis, following a singular or multiple attempts at cycle completion, it will exit the cell cycle into G0 with double the standard complement of DNA: a polyaneuploid cancer cell.

Though all treatments work in distinct ways, modern systemic therapies by design only target actively proliferating cells, or cells actively engaged in the cell cycle (Legesse-Miller et al., 2012). Therefore, an exit from the process of proliferation could be a viable survival tool for cancer cells (Blagosklonny, 2011). A cell within a non-proliferative state, and thus not cycling through mitosis, would not be affected by drugs designed to kill quickly dividing cells. And though the mechanism of quiescent cancer cells avoiding immune surveillance is still unclear, it is believed to be a mechanism similar to that of non-cycling stem cells that change their antigen presentation in the dormant state (Agudo et al., 2018). Hence, an exit from the proliferative state into a dormant G0 state makes these cells immunologically invisible and therapeutically untouchable (Leontieva and Blagosklonny, 2010). These G0 cells will continue to survive in a non-proliferative state, unaffected by both cancer treatments and the immune system of the host, until a more favorable environment for proliferation returns (Chen et al., 2016).

1.3. Polyaneuploid cancer cells (PACCs) arise from ROS induced cell-cycle arrest

Aneuploidy has been well-described in all cancer types, and is widely accepted as a distinguishing – and perhaps defining – characteristic of cancer (Taylor et al., 2018; Hanahan and Weinberg, 2011). Therefore, cancer cells that exit cell cycle at any point after S phase, including following aborted mitosis, are polyaneuploid cancer cells (PACCs). These cell-cycle arrested PACCs, also referred to as polyploid giant cancer cells (PGCCs) (Amend et al., 2019), that arise following initial treatment will likely survive any subsequent rounds of therapy. It is also theorized that in this state the cell could mutate further, leading to further advantage and capability for survival (Sharma et al., 2010). When these cells eventually re-enter the cell cycle, they will have the capacity to repopulate the tumor and lead to a cancer relapse (Stewart et al., 2007).

The presence of ROS and its damaging effects on DNA in cancer cells triggers the protective response of the DDR permitting the newly-formed polyaneuploid cells to escape into a protected G_0 state and avoid any further DNA damage (Fig. 3). (Srinivas et al., 2019; Ye et al., 2013) Shielded from therapy, these PACCs have the ability to not only survive, but re-emerge as therapeutically resistant parent cells capable of re-populating the tumor with resistant prodigy (Pienta et al., 2020).

PACC formation can be induced *in vitro* by the introduction of various stressors, such as chemotherapy (Fig. 4). (Pienta et al., 1557; Pienta et al., 2020) Anti-cancer therapy, coupled with the relentless oxidative stress stemming from therapeutically-dependent ROS, could induce and enrich PACC populations as they are the only cells capable of surviving the damaging effects. Furthermore, the increased ROS levels as both inherent of the cancer state and as result of therapy will benefit the resistant cancer cell's proliferation by upregulating cell signaling pathways, if able to re-enter the cell cycle (Perillo et al., 2020; Zhou et al., 1521; Liao et al., 2019; Szatrowski and Nathan; Schieber and Chandel, 2014). If PACCs are responsible for the introduction of phenotypic resistance seen in lethal cancers, applying anti-cancer therapies to metastatic cancer may induce a fully resistant phenotype (Pienta et al., 1557; Pienta et al., 2020).

CRediT authorship contribution statement

Morgan D. Kuczler: Conceptualization, Writing – original draft, Writing – review & editing. **Athen M. Olseen:** Conceptualization, Writing – original draft, Writing – review & editing. **Kenneth J. Pienta:** Writing – review & editing, Funding acquisition. **Sarah R. Amend:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

K.J. Pienta is a consultant for CUE Biopharma, Inc., is a founder and holds equity interest in Keystone Biopharma, Inc., and receives research support from Progenics, Inc. The other authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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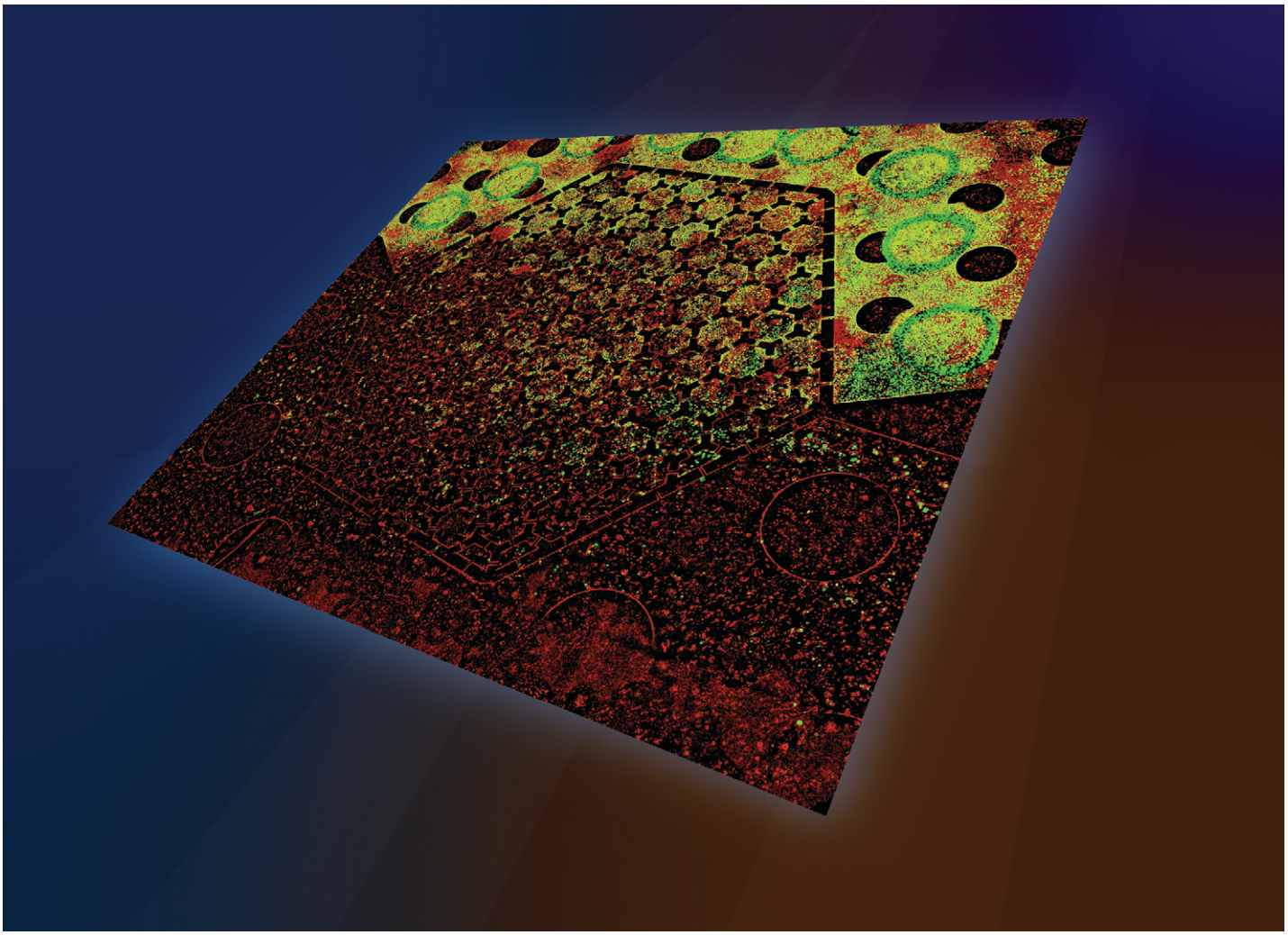

Showcasing research from Professor Austin's laboratory, Department of Physics, Princeton University, Princeton, NJ, USA

An *in vitro* tumor swamp model of heterogeneous cellular and chemotherapeutic landscapes

A microfluidic platform, the static evolutionary accelerator (EA), establishes a stable chemotherapeutic gradient across a topology of connected microhabitats *via* a purely diffusional mechanism. This adaptable, streamlined approach without an active media flow conveys a means to study the evolutionary and population dynamics of cancer while incorporating heterogeneities within the tumor microenvironment.

As featured in:

See Robert H. Austin *et al.*, *Lab Chip*, 2020, **20**, 2453.

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An *in vitro* tumor swamp model of heterogeneous cellular and chemotherapeutic landscapes†

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The heterogenous, highly metabolic stressed, poorly irrigated, solid tumor microenvironment – the tumor swamp – is widely recognized to play an important role in cancer progression as well as the development of therapeutic resistance. It is thus important to create realistic *in vitro* models within the therapeutic pipeline that can recapitulate the fundamental stress features of the tumor swamp. Here we describe a microfluidic system which generates a chemical gradient within connected microenvironments achieved through a static diffusion mechanism rather than active pumping. We show that the gradient can be stably maintained for over a week. Due to the accessibility and simplicity of the experimental platform, the system allows for not only well-controlled continuous studies of the interactions among various cell types at single-cell resolution, but also parallel experimentation for time-resolved downstream cellular assays on the time scale of weeks. This approach enables simple, compact implementation and is compatible with existing 6-well imaging technology for simultaneous experiments. As a proof-of-concept, we report the co-culture of a human bone marrow stromal cell line and a bone-metastatic prostate cancer cell line using the presented device, revealing on the same chip a transition in cancer cell survival as a function of drug concentration on the population level while exhibiting an enrichment of poly-aneuploid cancer cells (PACCs) as an evolutionary consequence of high stress. The device allows for the quantitative study of cancer cell dynamics on a stress landscape by real-time monitoring of various cell types with considerable experimental throughput.

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1 Introduction

Aggressively growing solid tumors rapidly outstrip the ability of the circulatory system to develop a coherent microcirulatory network around the tumor,[1] resulting in cancer cells within the tumor far from the characteristic diffusion length of ≈200 μm for resources within normal tissue.[2] This results in the generation of the internal "tumor" swamp, a region of the tumor with extreme metabolic stress.[3] There is also clear evidence of extensive interaction between cancer cells and other cancer cells, host stromal cells and cancer cells, as well as cancer-influenced host cells (*e.g.* cancer-associated fibroblasts, myofibroblasts, and macrophages) with other host cells within the tumor microenvironment.[4] The microenvironment is characterized by a profound heterogeneity in the distribution of chemotherapy, oxygen, or other resources.[5,6] Thus, solid tumors can be described within an ecological framework in which diffusion is the primary transporter of metabolites and chemotherapy, not the active hydrodynamic flow of normally irrigated tissue. In the context of a passive environment that applies strong selective pressures, the swamp presents opportunities for genetic or epigenetic changes crucial for cancer cell survival in environments where normal cells cannot survive.

Dynamics among various cell types within the tumor microenvironment have also been shown to affect the phenotypic behavior of cancer cells. These different cells also exist within the swamp. Traditional well-mixed cell culture provides a homogeneous environment that does not take into account the spatial complexities within the tumor swamp. Further, *in vivo* mouse models often lack the temporal resolution necessary to observe cellular processes.[7] Thus, more representative *ex vivo* models are required to recreate the adaptive tumor microenvironment in which cancer resides. Recent efforts have explored the potential for such models to develop patient-specific treatments.[8]

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An intuitive way of generating diffusive heterogeneity *in vitro* is to induce a purely diffusive chemical gradient within an artificial microenvironment, as chemical gradients typically hold key roles in regulating a number of cellular functions *in vivo* including migration, proliferation, differentiation, and carcinogenesis. Microfluidic devices have become a versatile platform for precise gradient control.[9,10] These gradient-generating devices can be broadly categorized into (i) constant flow generators which provide fixed boundary conditions,[11–13] and (ii) static generators which are solely based on diffusion.[14–16]

As illustrated in Fig. 1a, constant flow generators require an active pressure source to drive the media and create laminar flow in order to establish stable and time-independent chemical gradients within the microfluidic channels. Most of the existing flow-based gradient generators are associated with significant hydrodynamic shear stresses, which cause disruption of cell proliferation and affect cell morphology through various flow-mediated mechanisms such as mechanotransduction.[17] In contrast, as shown in Fig. 1b, static gradient generators establish a gradient without the presence of advection flow, offering a much more desirable physical environment for cell growth. However, static gradient generators require frequent media replenishment, which usually disrupts the distribution of chemical concentration through the period of an experiment.

Several attempts have been made to combine the advantages of flow-based devices and static gradient generators.[18–22] As shown in Fig. 1c, the media flowing channels and cell culture chambers are separated by slits which greatly increase the fluidic resistance and prevent shear stresses across cell culture chamber. As a result, long-term experiments can be done in the scale of several weeks. However, the application of these devices as pre-clinical research platforms remains limited, as the experimental setup is a complicated and labor-intensive process. Further, the need of active pressure sources such as a syringe-pump limits the experiment throughput.

Based on the philosophy of Sewall Wright, if a population of cells is split into a metapopulation and allowed to interact on a fitness landscape, the evolutionary dynamics of the system can be rapidly accelerated. From this principle, we have shown previously that these metapopulations, or weakly connected microenvironments, within a microfluidic device may induce rapid emergence of bacterial resistance to antibiotics as well as the formation of poly-aneuploid cancer cells (PACCs) in the context of chemotherapeutic resistance.[23–25] However, the previously reported microfluidic devices are designed based on the gradient generation mechanism demonstrated in Fig. 1c, which restricts the scalability of the experimentation platform.

In this paper, we describe a microfluidic device that constructs cancer cell metapopulations on a stress landscape yet does not require syringe pumps as active media sources as described previously.[21,25] The presented device, the static evolutionary accelerator (EA), generates a stable chemical gradient through diffusion between an on-chip pre-filled reservoir and an outer media buffer. This design overcomes major challenges faced throughout the development of the technology by successfully transitioning from a flow-based to a passive diffusion approach for gradient generation across the microhabitat array, enabling the ability to conduct long-term experiments with a much simpler, more cost-effective, and more seamless experimental setup in an effort to expand the use of microfluidic devices in preclinical cancer research.

As illustrated in Fig. 1d, the design of the chip allows the media in the outer buffer to be replenished without disturbing the chemical gradient. This is achieved since the reservoir is enclosed and filled up with fluid, such that the variation of fluid level in the outer open-ended reservoir caused by media

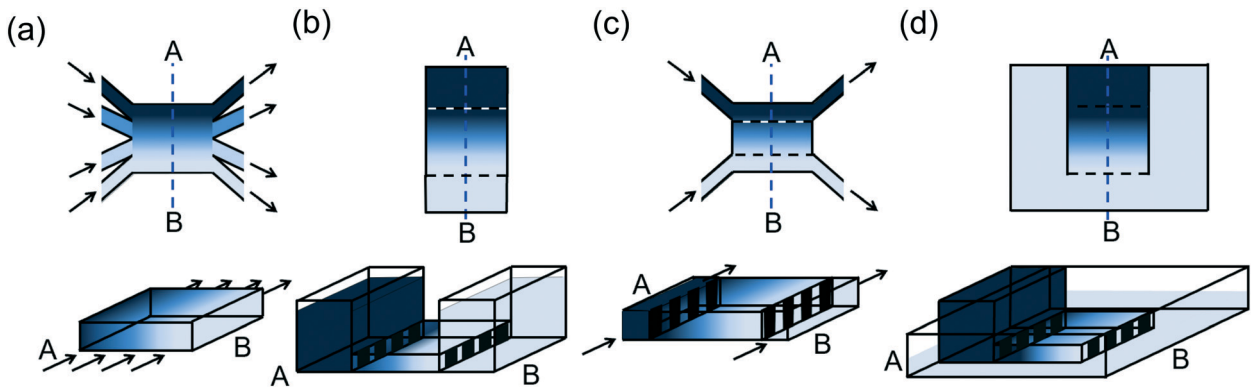

Fig. 1 Schematic diagrams of major microfluidic gradient generation mechanisms (top view and isometric view). (a) Constant flow gradient generators provide exceptional control over gradient precision and time efficiency. The hydrodynamic flow, however, leads to disruption of cell culture. For all diagrams, "A" and "B" denote opposing maximum and minimum chemical concentrations, as visualized by the shades of blue. (b) Static gradient generator with open-ended reservoirs offer a more desirable environment for cell growth, but are more suitable for short-term experiment since media replenishment could disrupt the distribution of chemical concentration. (c) Gradient generation through combination of laminar flow and diffusion resolves the issues as seen in (a) and (b). Nevertheless, the experimental setup could be complicated and labor-intensive. (d) A simplified schematic figure of the static gradient generator presented in this paper. With one enclosed reservoir and one open-ended reservoir, media in the outer buffer can be replenished without disturbing the chemical gradient. Long-term experiments can be performed without complicated tubing connections and bulky peripheral equipment for an active media source.

replenishment does not induce fluid advection. Without otherwise bulky peripheral equipment for an active media source, the experimental setup is greatly simplified and allows for high-throughput experimentation. Multiple experiments can be easily performed simultaneously, enabling numerous potential applications including but not limited to parallel experimentation for time-resolved downstream cellular assays, high-throughput drug screening, *ex vivo* modeling, and predictions for patient-specific treatments.

We validated the stability of the drug gradient within the static EA *via* COMSOL Multiphysics simulation and a physical gradient test with a fluorescent dye. We further cultured a bone-metastatic prostate cancer cell line of an epithelial phenotype (PC3-EPI) with a human bone marrow stromal cell line (HS-5) using the presented microfluidic static EA and observed cancer cell propagation for 6 days in the presence of a gradient of the chemotherapeutic docetaxel. This device has potential therapeutic implications as a robust *in vitro* platform for a wide variety of preclinical studies that better depict the human tumor microenvironment.

2 Experimental

2.1 Microfluidic chip design and fabrication

The microfluidic device pattern consists of hexagonal microenvironments in which cells can be cultured (Fig. 2a). The device, consisting of the pattern on the bottom and a reservoir to hold cell media, can be adhered to a 2-dimensional surface of cells (Fig. 2b and c). Cell media with drug can be filled on the outside, such that diffusion occurs inwards in one direction along the chip and also outwards from the reservoir (Fig. 2d). Diffusion fluxes between the source with no drug and the source with drug can balance, setting up a gradient of drug. Note that the operation of gradient generation can be reversed; *i.e.*, the media in the outer region may contain reagent of interest while the top reservoir contains normal growth media. Because there is no additional opening in the top reservoir, regardless of the height of outer buffer media level, there is no fluid advection in the device.

Specifically, the microfluidic device, made of polydimethylsiloxane (PDMS), consists of two compartments: the on-chip media reservoir and a PDMS microstructured chip with 109 hexagonal and 24 half-hexagonal interconnected chambers (Fig. 2a). The hexagonal chambers are 350 μm in side length. The floor of the lowest level of the PDMS device upon which the cells move is a 35 mm diameter hydrophilic 20-micron thick Lumox™ gas permeable film (Sarstedt, D 51588 Nmbrecht, Germany). Chemicals diffuse into the hexagonal chambers through the 15-micron wide and 180-micron long slits between the periphery channels and the hexagonal array. In order to create the device, a

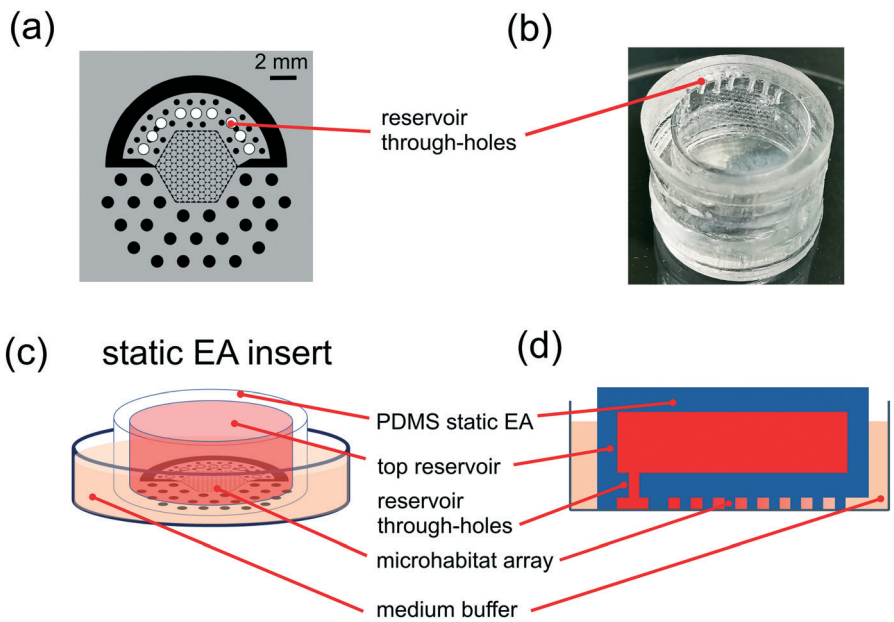

Fig. 2 Design and implementation of the static evolutionary accelerator (EA) insert. (a) Schematic design of hexagonal microhabitat array to be etched onto a silicon wafer for a depth of 100 μm. White circles within the design indicate punched through-holes connecting the microhabitat array to the on-chip media reservoir. Dark structures within the scheme illustrate the PDMS-casted walls that enclose the cells and support the device. Each side of each hexagonal microhabitat is 350 μm and the diameter of the entire chip is 21 mm. (b) Components of the PDMS-based static EA insert in practice. From top to bottom: micro-structured layer, capping layer, reservoir, and another capping layer. The microstructured layer is generated from soft lithography based on the silicon wafer patterned with the structure in (a). (c and d) Illustration of how gradients are generated from tilted side view and cross-section perspectives. The media in the top reservoir (red) and in the media buffer (orange) contain varying concentrations of the reagents of interest. Reagents diffuse across the hexagonal microhabitat array and generate 2D gradients within the cell culture region. The combination of the PDMS chip and the outer cell culture vessel is called a "static EA insert," which is placed in a customized sample holder described in Fig. 3.

silicon wafer is fabricated using standard photolithography (using light to pattern a thin film), deep reactive-ion etching (for 100 μm etch depth), and silanization. The PDMS (Sylgard 184 silicone elastomer kit) is mixed and poured onto the silicon wafer, degassed in a vacuum chamber, and further cured overnight at 60 °C. Afterwards, the PDMS chip is peeled from the wafer and through-holes are punched through the PDMS to allow fluid flow. The PDMS chip is then bonded with a reservoir and capping layers of PDMS (Fig. 2b) *via* oxygen plasma treatment. For gradient experiments (Fig. 5), black food coloring was added to the uncured PDMS to dissociate background fluorescence effects.

2.2 Cell culture platform and assembly

A stainless steel 6-well plate was designed to hold up to 6 Lumox™ dishes as well as 6 devices (Fig. 3a). Each static EA insert includes an opening for media replenishment, the top of which is covered by microadhesive sealer. The 6-well plate has double layer 35 mm glass windows underneath each Lumox™ dish to maintain thermal isolation and prevent water condensation. The plate can be set up during experiments lasting up to several weeks with suitable environmental factors such as an Ibidi thermal control unit that keeps the cells at 37 °C and a custom gas supply that mixes ambient air with the desired 5% CO_2. The gas supply system connects to the dish and pressurizes the Lumox™ membrane against the chip, ensuring sealing of the chip and the formation of the hexagonal microenvironments (Fig. 3b). The flow of normoxia gas can be regulated by gas valves and the gauge pressure is typically maintained at around 0.2 psi (1.4×10^4 Pa). The pieces of the six-well plate can be cleaned and reused by a standard solid autoclave procedure.

2.3 Device operation

Prior to the start of experimentation, the desired cell lines are detached with 0.05% trypsin/EDTA (Sigma-Aldrich) and pelleted. A total of 5×10^4 cells are cultured in each Lumox™ dish on the six-well plate. Cells are allowed to adhere in an incubator overnight. The next day, the PDMS devices are disinfected *via* UV exposure for 15–20 minutes and treated in an oxygen plasma system for around 30 seconds to create hydrophilicity at the microstructure layer. Immediately afterwards, pre-warmed culture media at 37 °C, degassed in a vacuum chamber for 5 minutes, is injected into the on-chip reservoirs *via* 22-gauge syringes. The degassing procedure of the media minimizes chances for entrapment of microbubbles within the structures on the chip. Note that this procedure sets up a gradient with the high drug region on the outside of the chip; alternatively, injected pre-warmed media with drug reverses the gradient.

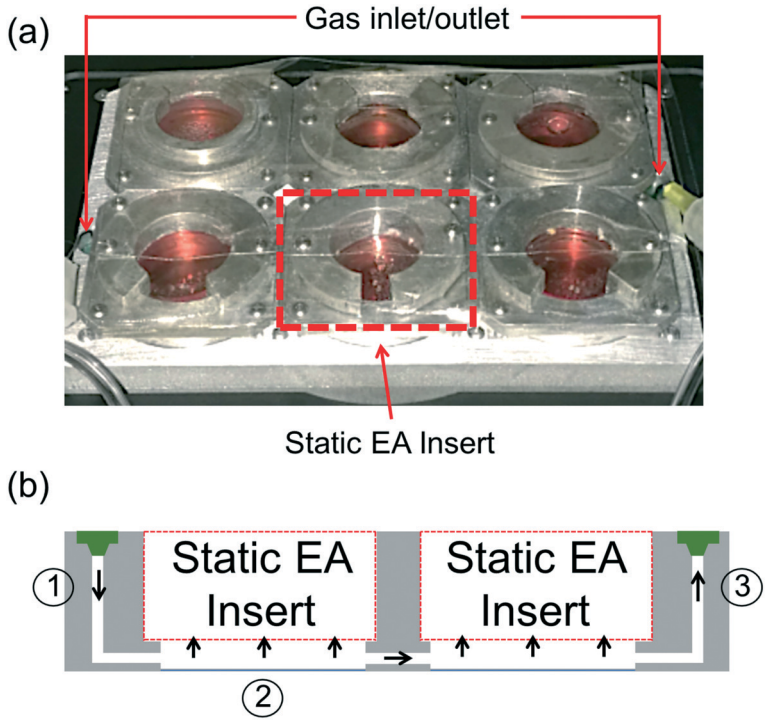

Fig. 3 Customizable setup allowing for long-term parallel experimentation. (a) Components of a six-well plate designed to hold up to six microfluidic chips to achieve parallelization. The solid red arrows indicate ports for gas supply influx and efflux, which uniformly pressurizes the chips. A static EA insert is indicated with a dotted red arrow. (b) Schematic of experimental setup, whereby the six-well plate including microfluidic chips is placed within an Ibidi incubator that regulates temperature (37 °C) and humidity (90%). The gas supply that pressurizes the Lumox™ dishes and seals the microfluidic chips is set to 5% CO_2, 20% O_2, and 75% N_2 and flows in through the port labeled 1, underneath the static EA inserts labeled 2, then out through a port labeled 3.

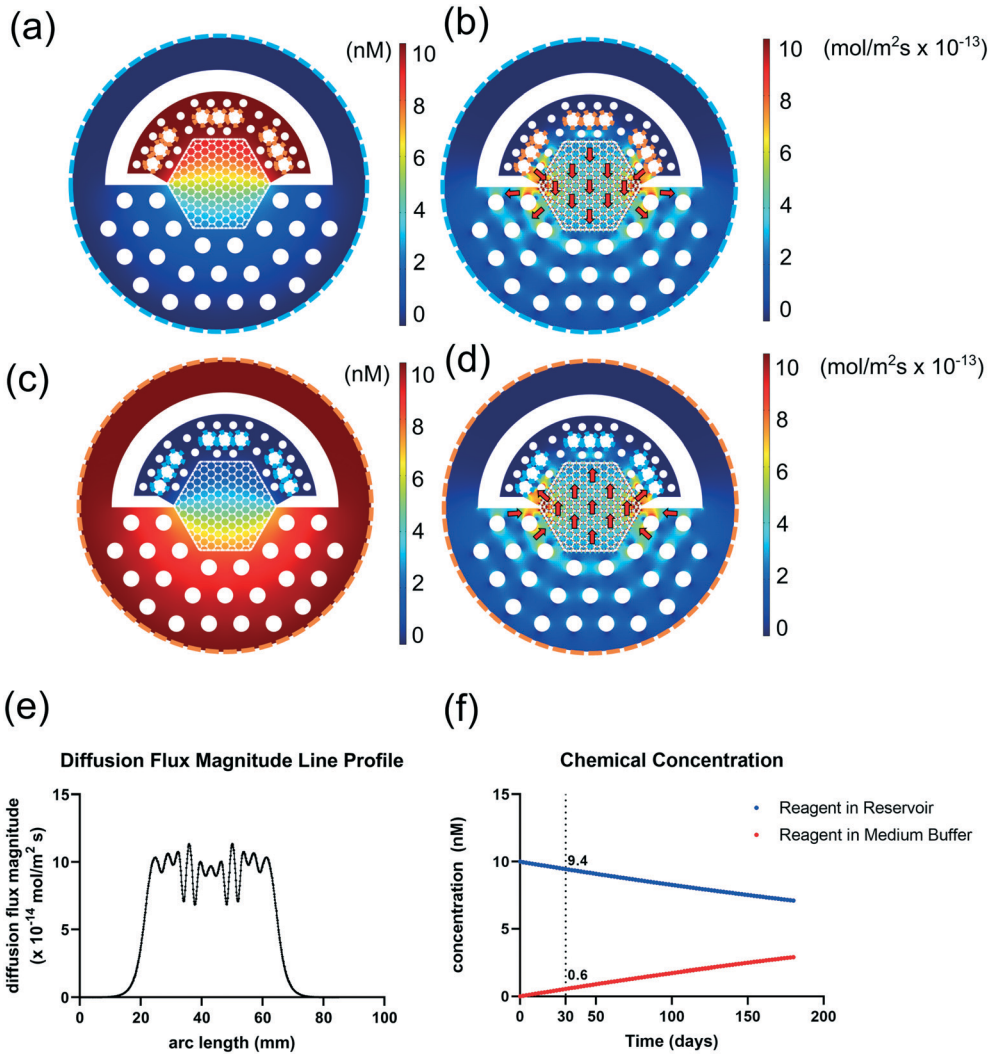

Fig. 4 COMSOL Multiphysics® simulation of the static diffusion mechanism and theoretically predicted gradients in microfluidic device. (a) Simulated reagent concentration distributions across the chip in the case where reagent diffuses outwards from the on-chip reservoir. Reagent diffuses from the nine circular through-holes at the top of the chip, into the hexagonal array, then diluted through the open boundary at the bottom of the array. Boundary conditions were set as 10 nM at the through-holes and 0 nM at the open boundary. (b) Magnitude of diffusion flux in the same simulation as in (a), with red arrows indicating direction of diffusion. The diffusion coefficient was set to be 10^{-9} m^2 s^{-1}. (c) Simulated reagent concentrations across the chip with reagent diffusing inwards from the outer region, with normal media in the on-chip reservoir. Boundary conditions were set as 0 nM at the through-holes and 10 nM at the open boundary. (d) Magnitude of diffusion flux in the same simulation as in (c), with red arrows indicating direction of diffusion. The diffusion coefficient was set to be 10^{-9} m^2 s^{-1}. (e) Line profile of diffusion flux magnitude around the entirety of the chip boundary. (f) Concentration of reagent over time depending on where the reagent is located. Within 30 days, the concentration of the reagent in either the reservoir or the media buffer changes by around 6%, which is robust enough for practical experimental purposes.

To initiate the experiment, the media within the Lumox™ dishes (with cells adhered) is replaced by 1 mL of prewarmed and degassed media. Chips are then placed directly on top of the gas-permeable Lumox™ membranes by approaching the surface at a 15° angle to minimize bubble formation. Each chip is then clamped into place onto the culture dish and a sheet of microadhesive sealer is spread over the top of the PDMS chip to minimize media evaporation. The entire six-well plate is placed into an on-stage incubator on the motorized stage of an inverted epifluorescence microscope. The gas supply pressurizes the Lumox™ membranes against the PDMS chip to ensure sealing of the device. Media with drug can be replaced every 2 days by extracting and injecting 1 mL directly through the microadhesive sealer from or into the area surrounding the PDMS chip. The reagent of interest then diffuses into the hexagonal cell culture region, setting up a gradient with the normal culture media diffusing out through the through-holes on the reservoir.

2.4 Data acquisition and image processing

To generate population data with sufficient temporal resolution, we utilized in this paper time-lapse image acquisition on an inverted epifluorescence microscope with a

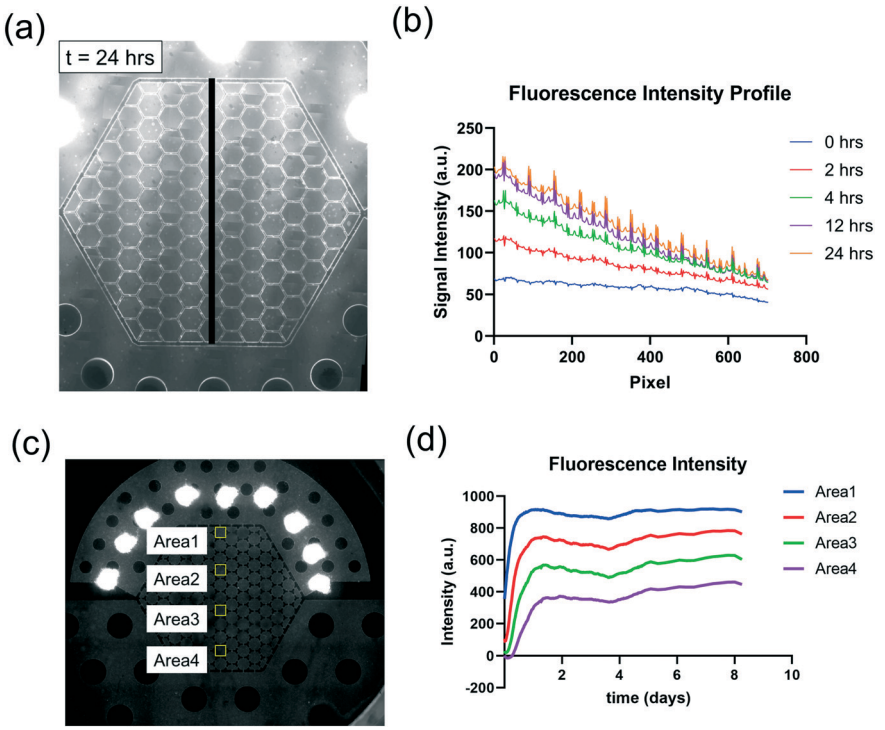

Fig. 5 Verification of gradient generation across multiple days. (a) A static EA with reservoir filled with 0.1 mM of rhodamine-6G, a fluorescent dye, at t = 24 hours post-installation. The marked black line indicates the fluorescence intensity line profile measured vertically across the chip. (b) Line profiles of fluorescence intensity as shown across the chip in (a) at various time points. The gradient stabilizes at around 24 hours. Peaks in the profile reflect uneven changes in the intensity as the border of a microhabitat is crossed. (c) Observation of four indicated areas on the static EA, with reservoir filled with rhodamine-6G dye. (d) Longitudinal time profile of the four areas in (c) across 8 days, indicating that intensity and corresponding gradient remain stable across the device and across time.

motorized x–y stage, focus knob, shutter, and filter cubes. Fluorescence images may also be taken manually at desired time points. In our experiments, we employed the Nikon NIS-Elements software to acquire stitched images across various channels at 10× magnification with autofocus routines. Images were taken every hour, with experiments possessing the ability to run on the time scale of weeks.

After the experiment, images were converted to TIFF format and compressed (Fiji/ImageJ software). Fluorescence intensities were quantified *via* ImageJ and relevant algorithms such as background subtraction and particle analysis to determine cell number were performed (ESI†). MATLAB ver. R2019a and Graphpad Prism 8.3.0 were employed for graphical output.

3 Results and discussion

3.1 Demonstration of gradient generation

We modeled the process of gradient formation numerically *via* the diffusion equation with fixed boundary conditions (COMSOL Multiphysics). The COMSOL simulation demonstrates the distribution of reagent concentration (Fig. 4a) and magnitude of diffusion flux (Fig. 4b) in the static EA at steady state, with the diffusion coefficient set to be 10^{-9} m^2 s^{-1} and boundary conditions of 0 nM and 10 nM. We consider two scenarios: 1) the 10 nM reagent diffuses out from the top reservoir, into the hexagonal array, and then is diluted at the open boundary at the bottom, and 2) the 10 nM reagent is added in the outer region/media buffer, diffuses into the hexagonal array, then diluted by the on-chip reservoir. The results of these two cases (Fig. 4a–d) are symmetrical. The line profile of the diffusion flux magnitude around the circular outer boundary is plotted in Fig. 4e. The total loss of reagent is equal to the integral of the diffusion flux magnitude over the corresponding surface area, which gives the dissipation of the reagent per second as 4.4×10^{-19} mol s^{-1}. Note that the total amount of reagent in the reservoir or media buffer is 2×10^{-11} mol such that the dissipation of the reagent is negligible within the period of the experiment (Fig. 4f). Therefore, during the experiment, we may assume the concentration of reagent stays constant on the boundary.

Compared with flow-driven gradient generators, this static diffusion mechanism requires a longer time to establish the concentration profile pattern. To measure the timescale of gradient generation and to validate the predictions of our simulations, we performed gradient tests with a fluorescent hydrophilic small-molecule organic dye, rhodamine-6G. The reservoir of the static EA was filled up with 0.1 mM rhodamine-6G. In order to block the background fluorescent signal from the rhodamine-6G within the reservoir, we employed a black PDMS chip by mixing black food-dye into the PDMS elastomer prior to the curing process. The gradient

of fluorescence intensity, which is directly proportional to the concentration of rhodamine-6G, increased monotonically and reached equilibrium by 24 hours after chip installation (Fig. 5a and b). In a separate experiment, four indicated areas on the chip showed that a stable linear gradient is established through 8 days after the start of the experiment, once normalized for increases in background light intensity (Fig. 5c and d).

We also investigated the diffusion of rhodamine-6G into PDMS as a potential source of error, as PDMS is known to absorb small chemical molecules.[26,27] In our case, we typically work with small molecules which should not have high diffusion constants within a well-cured (24 hours at 60 °C) PDMS polymer. However, to test this assumption, we measured the increase in fluorescent intensity of a sealed PDMS pillar over time within the experiment described in Fig. 5c (Fig. 6a). We can obtain a rough estimate of the upper bound of the diffusion coefficient of the charged dye (rhodamine-6G) through PDMS by considering the 1D diffusion equation

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2},$$

where intensity $I \sim c$. The pillar is around 760 μm in length and thus $\Delta x \approx 380$ μm. We take maximum fluctuations of around 100 units of intensity across the pillar, and employ 1 unit of intensity per day as $\frac{\partial c}{\partial t}$ by taking the standard error of the slope of the best-fit line to the linear portion of Fig. 6b. This calculation assumes all fluctuations in observed intensity across the pillar are attributed to diffusional effects and therefore correspond to an upper bound. We find that diffusion effects of the dye into the PDMS is negligible after controlling for background noise, with an calculated upper bound for the diffusion coefficient of rhodamine-6G into PDMS as 1.7×10^{-10} cm^2 s^{-1}, a very small number compared to the diffusion of the dye in water, which is on the order of 10^{-5} cm^2 s^{-1} (Fig. 6c and d). Therefore, we do not believe that small molecule diffusion through PDMS significantly influences the static gradient. Nevertheless, given the evidence suggesting small molecule absorption by PDMS with relevance to cell culture,[26,27] the presented static EA may be modified in further studies *via* surface coatings or other replacement polymers in order to increase device applicability. In our device, the lack of an active source (pump) guarantees bubble-free and static fluid dynamics, which ensures stable chemical gradient across the chip. This pump-free configuration is compatible with commercially-sized well plates and allows for much higher experiment throughput. The aforementioned advantages allow the cell culture device to be an ideal platform to investigate the population dynamics of cancer cells in a chemotherapy-induced stress landscape.

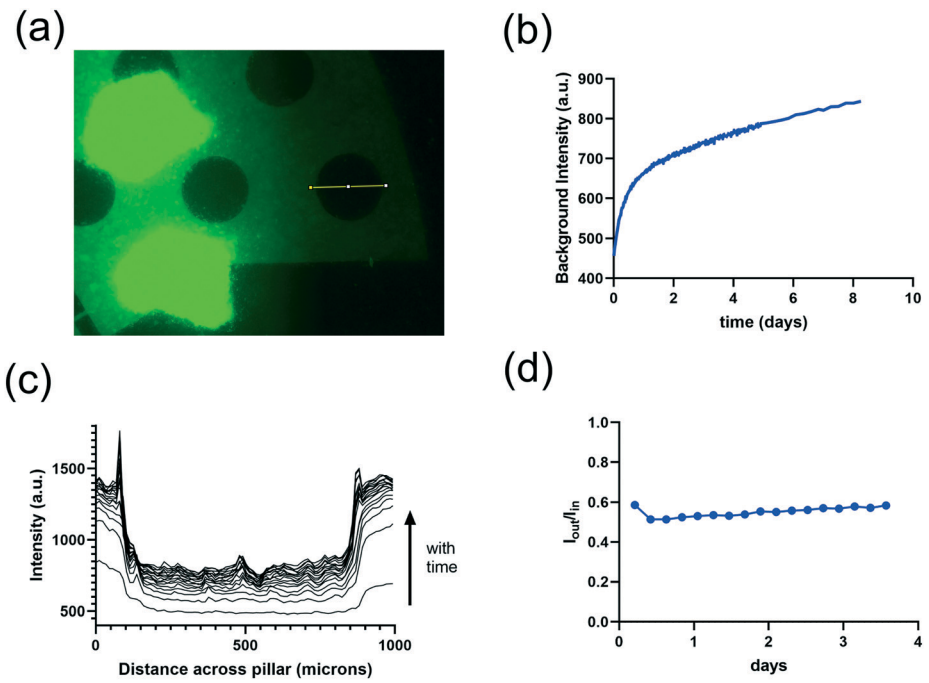

Fig. 6 Insignificant contribution of diffusion of dye through PDMS to the small molecule gradient. (a) Analysis is focused on the pillar on PDMS indicated with a yellow line. This pillar is sealed to the Lumox™ membrane with dye occupying the space surrounding the pillar. (b) Increase in the intensity of the center of the pillar over the course of an 8 day experiment. (c) Line profile of the pillar in part (a) over time. The curves generally reveal a uniform profile within the pillar at any particular point in time, with the average intensity increasing quickly then leveling off. Since there is always a uniform profile within the pillar, there are negligible diffusional effects. (d) Ratio of the intensity outside the pillar I_{out} (first point in each profile in part (c)) to the average intensity within the pillar I_{in} over time. The ratio is relatively conserved through time, implying that general increases in intensity are background noise effects and do not affect I_{out} or I_{in} in varying ways.

3.2 Population dynamics of bone marrow stroma and cancer cells

To determine the capability of the device to provide suitable conditions for cellular propagation under a chemotherapeutic gradient over long time scales, PC3-EPI, an E-cadherin/CDH1-positive/vimentin-negative PC3 clone with epithelial phenotype described previously,[25] was co-cultured with HS5, a human bone marrow fibroblast stromal cell line (ATCC). PC3-EPI was engineered *via* lentiviral vector to possess nuclear histone fluorescence with green fluorescent protein (GFP) and constitutive cytoplasmic red fluorescence with mCherry.[25] Docetaxel was used to generate gradients of 0 to up to 25 nM across the static EA. Employing the platform as described in Fig. 3, we performed 6 simultaneous experiments that included gradient verification, no gradient, and varying gradient conditions (ESI† Fig. S1). Real-time imaging of cells was conducted with three different channels, brightfield, mCherry, and GFP, across the entirety of each static EA at 10× magnification every hour. Images were stitched together in the NIS-Elements software, and analysis was conducted after importing to ImageJ/Fiji software. Image processing and cell counting were achieved with a custom protocol and set of algorithms (ESI† Fig. S2). Movies of relevant gradient and control experiments are provided in the ESI.†

We describe a representative experiment in Fig. 7, which shows the progression of PC3-EPI cancer cells and HS5 stromal cells within the static EA chips under the conditions of either no docetaxel present or a 15 nM gradient of docetaxel, with cancer cell nuclei colored as green (GFP fluorescence) and stromal cells colored red (processed brightfield). The gradient is established with the high-drug region at the bottom of the chip (Fig. 7d–f). To better understand these dynamics, we quantified these cell count data and demonstrate population behavior in Fig. 8. In particular, we consider the cancer population proportion p_γ, defined as

$$p_\gamma = \frac{\gamma}{\gamma + s},$$

where γ and s are the cell counts for PC3-EPI and HS5, respectively. In the control condition without docetaxel, PC3-EPI cancer cells grew homogenously across the microhabitats to reach high confluence and population dominance by 3 days (Fig. 8a–d, ESI† Fig. S3), with the time scale of growth comparable to previous experiments with pump-driven devices.[21] However, within the 15 nM gradient experiment, with originally relatively uniform populations of both cancer and stromal cells, by day 3, a marked transition in cancer cell population fraction occurs, revealing differential effects of

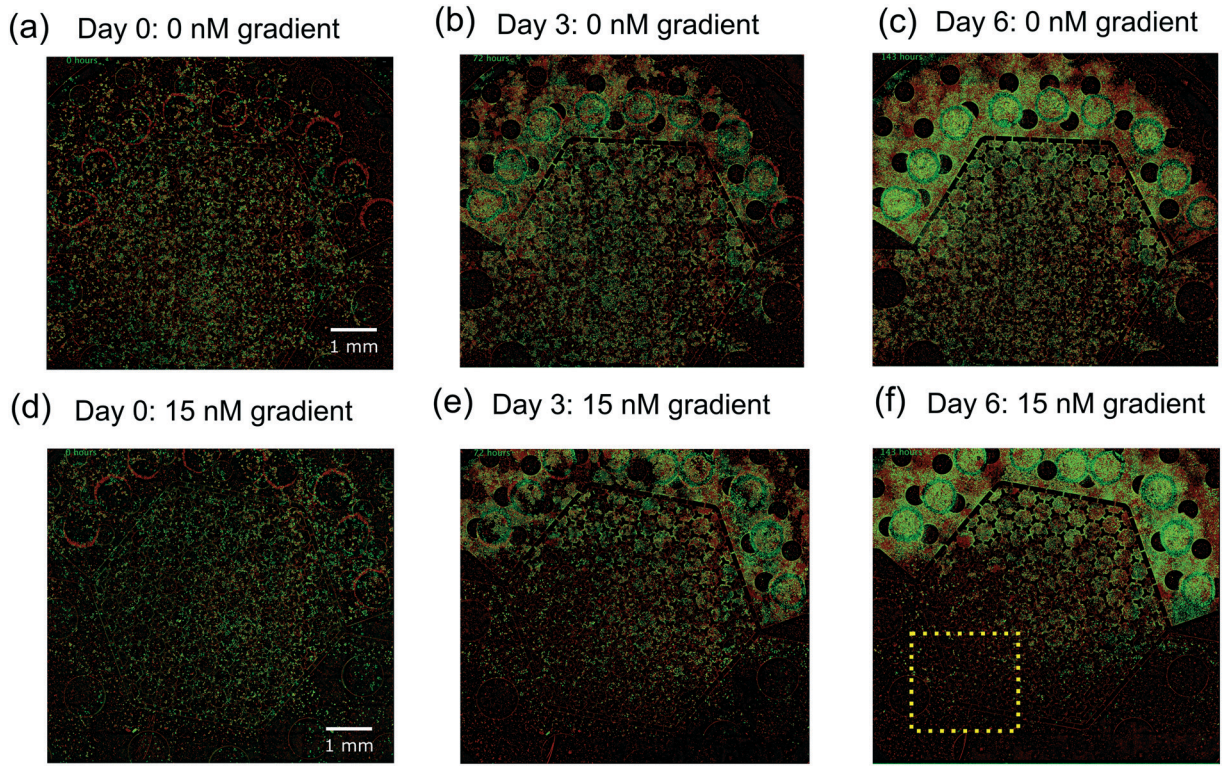

Fig. 7 Visualization of proof-of-principle coculture of PC3-EPI (nuclear GFP stain, colored as green) and HS-5 (processed brightfield, colored as red) without drug and with docetaxel gradient (0 to 15 nM). (a–c) Co-culture of PC3-EPI and HS-5 without drug in a control experiment, with images shown on days 0, 3, and 6. The scale bar shown in (a) applies to both (b) and (c). PC3-EPI cells achieved confluency by day 3. (d–f) Co-culture of PC3-EPI and HS-5 with docetaxel in a gradient ranging from 15 nM (outside region) to 0 nM (on-chip reservoir), at days 0, 3, and 6. The scale bar shown in (d) applies to both (e) and (f).

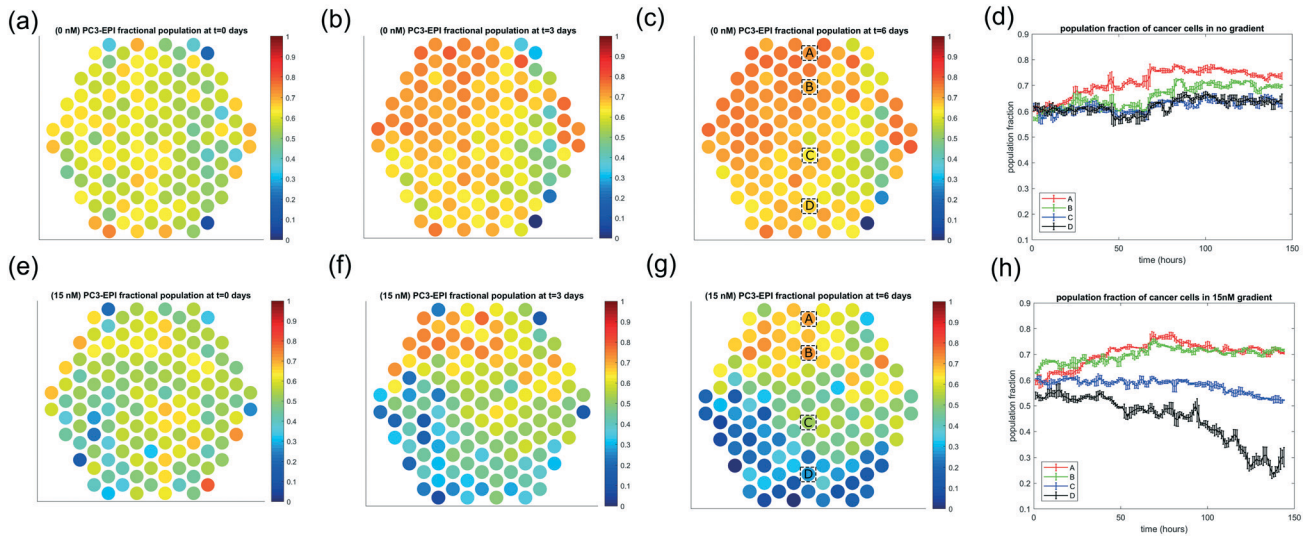

Fig. 8 Population spatial distribution of cancer PC3-EPI for control and gradient experiments. (a) Analysis performed on images such as those shown in Fig. 7 to determine the population fraction of cancer cells in a control experiment with no drug gradient at t = 0 days. The population fraction is charted as a heatmap within the hexagonal array, with each circle corresponding to a microhabitat in the static EA. Each colored dot follows the range depicted in the color bar to the right representing the average PC3-EPI fractional population calculated for each representative time point for both the drug gradient (15 nM) and control (0 nM) experimental series. (b) Similar plot as in (a) but at t = 3 days. (c) Similar plot as in (a) but at t = 6 days. (d) Time evolution of cancer population fraction for the control experiment across four distinct areas of the EA chip (A–D as shown in (c)). Data are displayed as a moving mean with n = 3. Error bars correspond to the mean ± moving standard deviation of adjacent 3 data points. (e) Population fraction of cancer cells in a representative experiment with a 15 nM docetaxel gradient at t = 0 days. The drug gradient is established from the bottom of the chip (high-drug area) to the top of the chip (low-drug area). (f) Similar plot as in (e) but at t = 3 days. (g) Similar plot as in (f) but at t = 6 days. (h) Time evolution of cancer population fraction for 15 nM gradient experiment at the same areas of the EA chip (A–D as shown in (g)), where data are displayed with moving mean (n = 3) ± moving standard deviation (n = 3).

docetaxel as a function of drug concentration (Fig. 8e–h, ESI† Fig. S3). The continuous transition in population dominance of cancer cells is maintained and magnified through 6 days of experimentation (Fig. 8g). The population fractions plotted in Fig. 8d and h correspond to the time course of particular microhabitats denoted in Fig. 8c and g. The curves for both Fig. 8d and h are relatively smooth, and the gradual transition from growth to failure to survive suggests that,

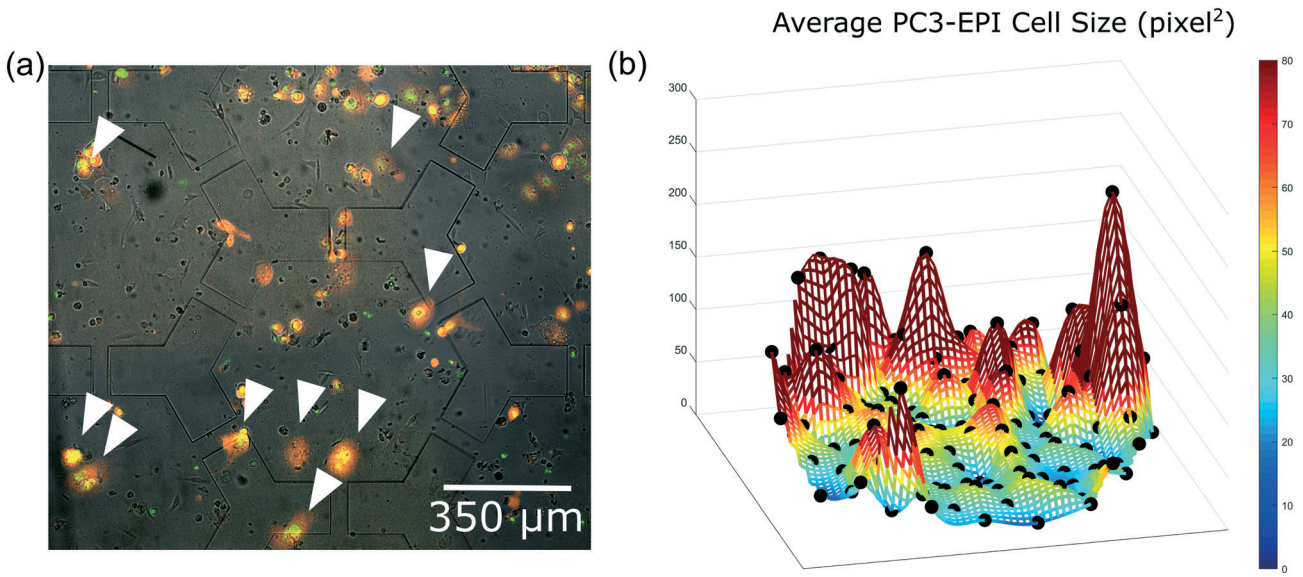

Fig. 9 Exemplification of evolutionary phenomenon of emergence of poly-aneuploid cancer cells (PACCs) in high-stress areas. (a) PACCs are indicated with white arrows. Brightfield, red, and green channels overlay capturing non-labeled stromal cells only visible in pure brightfield and PC3-EPI fluorescently-labeled nuclear and cytoplasmic cellular components of each cell displayed respectively in the red and green channels. Image taken from boxed region from Fig. 7f for day 6 within the 15 nM gradient. (b) Average PC3 cell size per microhabitat across the chip for day 6 populations as in Fig. 8g based on percentage area coverage by cancer cell cytoplasm.

locally, the drug concentration has a monotonically increasing negative effect on cancer cell proliferation. The observation of the transition in cancer fractional population dominance was similarly noted across separate experiments (ESI† Movies).

Notably, whereas on the population level, cancer cells fail to survive in high-drug areas, we observe that among the cancer cells that do survive, many of the cells consistently surviving in areas of high stress exhibit an enrichment of the poly-aneuploid phenotype with enlarged cell size and either an enlarged nucleus or multiple nuclei (Fig. 9a), corroborating the results of Lin *et al.* (2019).[25] Accordingly, the average cell size per microhabitat increases across the chip within the 15 nM gradient experiment at 6 days, suggesting an enrichment of these cells at preferentially higher drug concentration under the assumption that phenotypically larger cells correlate with a poly-aneuploidy subpopulation (Fig. 9b). Studies have reported previously the association between higher DNA content ($>4n$) and cell volume, both in the context of eukaryotic cells in general and in cancer.[28–30] In particular, we have previously shown that large size and polyploidy are key defining features of PACCs and have demonstrated that when PACCs are formed, either by fusion or failed cytokinesis, that their size is increased.[25,31] Future study will focus on the mechanism of evolution of these resistant cells, their characterization *via* downstream analysis, and the potential role of stroma in mediating resistance.

4 Conclusions

In this paper, we have designed a static microfluidic-based cancer-on-chip device with the ability to generate stable and controlled gradients of chemotherapy across connected microenvironments. This system presents a novel static gradient generation method, alleviating the need for pump-driven flow-based methods, significantly reducing experimental workload and increasing throughput. We modeled computationally the formation of a two-dimensional gradient on the time scale of weeks and validated the ability for the microfluidic static EA to maintain this gradient utilizing fluorescent dyes.

There is a need for more representative *in vitro* models to reproduce the tumor microenvironment prior to animal models in the drug development pipeline in order for better prediction of cancer progression as well as responses to drugs within dynamical stress landscapes. The cooperative interactions between tumor cells and the surrounding environment have been well-characterized as survival strategies against drugs.[32,33] Specifically, prostate cancer is well-known for predominantly metastasizing into bone marrow. The bone marrow provides a conducive environment for metastasized prostate cancer, allowing the cancer cells to stay dormant for a period of time before transitioning into the proliferating phase.[34,35] The process of how and why prostate cancer can be triggered to exit dormancy state and proceed to subsequent secondary tumor growth remains poorly understood, requiring further investigation.

Our evolutionary accelerator (EA) device offers a robust, high-throughput method to recapitulate elements of the complex *in vivo* microenvironment by allowing for multiple cell types to interact on a fitness landscape, driven by faster evolution in a low-N limit of isolated but connected populations. In comparison to other platforms to probe the roles of tumor–stroma interactions in chemotherapeutic resistance,[36] this platform importantly incorporates stress heterogeneity with a drug gradient, better representing the drug-induced evolutionary outcomes within the tumor microenvironment. We have previously described that in such gradient systems with weakly connected microhabitats, cells that become stress-resistant or mutants in one area can migrate to areas of even higher stress since they will possess greater fitness relative to other cells.[25] Since the total number of cells is greatly decreased in areas of higher stress, the mutants can more easily establish themselves within that new habitat.[25] Overall, the static EA reflects both population- and environmental-level heterogeneities.

As a proof-of-principle demonstration of our microfluidic system, we studied the co-culture of PC3-EPI prostate cancer cells with HS5 human bone marrow cells under a gradient of docetaxel. We employed extended time-lapse imaging on an on-stage incubator with high spatial and temporal resolution. We found a continuous transition in cancer cell proliferation and survival across the same chip on a population level, both in terms of raw cell count and relative fractional population when compared to the stromal cells. We further observe the emergence of large poly-aneuploid cancer cells at high-drug regions on the chip as a consequence of drug-induced genomic instability. We note that, within our technology, nutrient content within media diffuses uniformly throughout the hexagonal microhabitat array in theory; nevertheless, more validation needs to be conducted to ensure that cellular dynamics are not affected by local metabolite depletion within the microenvironments. If necessary, channel sizes could be modulated accordingly to provide for greater nutrient diffusion flux magnitudes. Moreover, the precision of the stromal cell automated algorithmic counting faces limitations due to the nonuniformity of brightfield imaging but can be alleviated by employing fluorescently labeled stromal cell lines. Further work will involve downstream sequencing analysis of resistant subpopulations and an exploration of the role of cell migration in the evolutionary dynamics.

This device will be important to future studies on the complex interactions between multiple cell types in a heterogeneous micro-ecological stress landscape. We hope that this work will act as a more robust preclinical platform for drug development and contribute to better understanding of how issues such as drug heterogeneity may have implications for tuning of chemotherapy scheduling, drug resistance, or recurrence.

Author contributions

Conceptualization: KL, YS, JS, KP, JQ, RA. Formal analysis: YS, KL. Investigation: KL, YS, GT, KM, PS, YZ, RA. Software: KL, YS. Supervision: RA, JS. Resources: SA, KP. Methodology: KL, YS, RA. Writing – original draft: YS, KL, RA. Writing – review & editing: YS, KL, RA. All authors read and approved the final draft.

Conflicts of interest

There are no conflicts to declare.

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Cancer recurrence and lethality are enabled by enhanced survival and reversible cell cycle arrest of polyaneuploid cells

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We present a unifying theory to explain cancer recurrence, therapeutic resistance, and lethality. The basis of this theory is the formation of simultaneously polyploid and aneuploid cancer cells, polyaneuploid cancer cells (PACCs), that avoid the toxic effects of systemic therapy by entering a state of cell cycle arrest. The theory is independent of which of the classically associated oncogenic mutations have already occurred. PACCs have been generally disregarded as senescent or dying cells. Our theory states that therapeutic resistance is driven by PACC formation that is enabled by accessing a polyploid program that allows an aneuploid cancer cell to double its genomic content, followed by entry into a nondividing cell state to protect DNA integrity and ensure cell survival. Upon removal of stress, e.g., chemotherapy, PACCs undergo depolyploidization and generate resistant progeny that make up the bulk of cancer cells within a tumor.

metastasis | drug resistance | tumor microenvironment | whole-genome doubling | evolution

It is widely recognized that the majority of cancer cells within a tumor have an abnormal number of chromosomes, i.e., are aneuploid (1–4). A polyaneuploid cancer cell (PACC) is an aneuploid cancer cell that has undergone whole-genome doubling (WGD), resulting in at least twice the complement of the original aneuploid genomic content. Unusually large aneuploid cancer cells have been documented in the cancer literature since 1858 when they were first described by Virchow (5–38). These giant aneuploid cells, observed in cell culture and pathologic tissues from patients, have been generally disregarded as not functionally important: irreversibly senescent or destined for mitotic catastrophe, too morphologically misshapen, and with chromatin too disorganized to directly contribute to tumorigenesis.

Recent evidence demonstrates that PACCs are viable and exist as a distinct and functional cancer cell state, able to resist stress within the tumor microenvironment (5–31). This allows us to develop a unifying theory to explain cancer recurrence and lethality that builds upon and unifies the observations of multiple fields of study, including aneuploidy, stem cell biology, genetic instability, tumor cell heterogeneity, senescence, quiescence, therapeutic resistance, and giant cells. The "hallmarks of cancer" provide the framework for tumorigenesis, but the hallmarks do not explain therapeutic resistance, recurrence, or lethality (39). The basis of our theory is that the formation of the PACC state in response to microenvironmental and therapeutic stress enables resistance to systemic cancer therapy

Metastatic cancer is ultimately resistant to virtually all systemic therapies and continues to kill more than 10 million people per year around the world (40–42). This suggests a common mechanism for cancer resistance that evolves convergently in 10 million people each year, regardless of the driver mutation or the tissue of origin (42, 43). Resistance to therapeutic interventions has classically been attributed to genetic tumor cell heterogeneity: Among the billions of cancer

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cells in a tumor, mutations lead to at least one cancer cell becoming resistant to a particular therapy (44–65). Since lethal cancer demonstrates resistance to therapeutic agents that it has previously not been exposed to, particular resistance mutations appear to develop by stochastic chance, fueled by aneuploidy (an abnormal number of chromosomes) and genetic instability (32, 44, 60, 61, 66–68). In the classic view, resistance to each different therapy requires that the appropriate mutations that confer the different versions of resistance are acquired by at least one cell. Newer models have found potential evidence for the gradual, multifactorial adaptation to the inhibitors through acquisition of multiple cooperating genetic and epigenetic adaptive changes of multiple partially resistant clones (69). Another alternative model of therapy resistance is the cancer stem cell model, in which a rare therapy-resistant population of cancer stem cells give rise to a recurrent population (36, 70–72).

In contrast, we theorize that lethal cancer is mediated through the generation of PACCs. PACCs form by utilizing evolutionary and developmental programs that utilize variations of the canonical cell cycle that allow bypass of mitosis and/or cytokinesis and permit entry into a polyploid state (73–78). These polyploidization programs are commonly observed as transient defense mechanisms in other organisms and in human tissue as an adaptation to environmental or metabolic stressors. For example, evolutionary programs for polyploidization have for eons enabled both unicellular and multicellular organisms a transient defense against toxins by entry into a nonproliferative state. Over the evolutionary history of life, polyploid programs have led to several events of persistent WGD, i.e., species polyploidy, in which genetic alterations associate with survival adaptations. The PACC theory of therapeutic resistance and cancer recurrence accounts for the ubiquity of recurrence in three steps (Fig. 1). First, a few tumor cells respond to stress (e.g., chemotherapy or metabolic stress as a consequence of uncontrolled growth) by accessing alternate cell cycle programs that form PACCs. Second, as part of cell enlargement and the polyploid program, a PACC pauses cell division, which allows it to adapt to toxic environments while protecting its DNA. This state also provides increased cell resiliency and survival in foreign secondary sites, enabling successful metastatic seeding. Third, when the stressor is removed, the PACC can undergo depolyploidization and reinitiate cancer cell proliferation. The resulting cells resume the cell cycle and now carry novel adaptations for overcoming the host defenses as represented in the hallmarks of cancer (Fig. 1).

Cells that have the capacity to form PACCs can alter their cell cycle to bypass mitosis and/or cytokinesis to become polyploid and are therefore not shunted to apoptosis and consequently exhibit enhanced survival under conditions of stress (79). The associated increase in cell size and concomitant decrease in surface-to-volume ratio may either protect the cell from local environmental damage by limiting the overall toxin load within the cell or assist the cell to broaden its environment for the scavenging of oxidants and nutrients. Simultaneously, the increase in genomic material resulting from polyploidization may provide building blocks for increased RNA and protein synthesis that provide raw materials for increased cellular metabolism, detoxification, and extended dormancy. In addition, the increase in genomic material may enable the cell to either avoid lethal genomic damage secondary to extra copies of genes, increase heritable variation, or allow self-genetic modification that allows new functionality or selection of robust progeny.

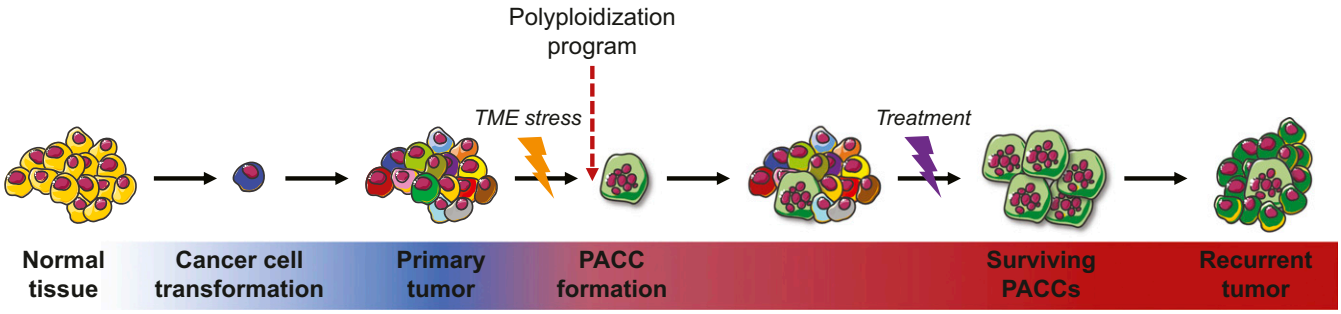
PACCs

PACCs exist as a distinct state of viable cancer cells, unique in that they contain multiple full copies of their aneuploid genome (5–38). The terminology for these cells has varied, including polyploid giant cancer cells, multinucleated giant cancer cells, blastomere-like cancer cells, osteoclast-like cancer cells, pleomorphic cancer cells, large cancer stem cells, and PACCs. Growing evidence now suggests that PACCs are functional actuators of therapeutic resistance. Notably, PACCs are observed in cancer cell lines (Fig. 2 *A.1* and *A.2*), in animal models (Fig. 2*B*), and in patients (Fig. 2*C*) across virtually all tumor types (5–31). Our growing insight into the role of PACCs in tumor progression underscores the importance of defining their biology, function, and relevance to cancer lethality. Important open questions concern how and when PACCs arise during tumorigenesis, how PACCs serve an adaptive role for cancer cells, and how they contribute to cancer's lethality.

Here, we offer a unifying theory for these three questions. Our theory relies on emerging data on how therapeutic resistance arises from PACCs. In this model, resistance is mediated through access to evolutionary and developmental programs for polyploidy that increase cell size and contents, including DNA. The increased cell size and organelle contents allow increased capacity for metabolism as well as for mitigating toxic stressors such as reactive oxygen species (63, 80, 81). Cancer cells with the ability to form PACCs exit the cell cycle and avoid DNA damage, potentially providing a universal mechanism of therapeutic resistance that is mutation (and therefore therapy) agnostic. This process shares many similarities with the phenomenon of therapy-induced reversible senescence (23, 26, 34, 36, 82–87). Further investigations are required to understand how cell cycle exit, quiescence, and senescence are related (87–89). While exiting the cell cycle and maintaining quiescence until the systemic stress has passed may be enough to allow repopulation of the 2N+ cancer cell population, access to greater genomic material could also mediate resistance in PACC progeny. The access to greater amounts of genetic material promotes genomic stability through extra copies of genes while simultaneously allowing for the potential generation of resistant progeny through either diverse mutations (e.g., gene duplication, chromosomal rearrangements) or via self-genetic modification prior to producing progeny. This access to increased heritable variation may be dispensed to their 2N+ aneuploid progeny that make up the bulk of cancer cells within a tumor, providing ecological and evolutionary rescue to otherwise-doomed populations (5, 16, 29, 35, 37, 39, 90–95). PACCs, therefore, share many similarities with cancer stem cells (16, 48, 96, 97). Defining the stem cell properties of PACCs and the mechanisms by which PACCs generate 2N+ progeny requires further investigation.

Cancer Cells Access Evolutionary and Developmental Polyploid Programs to Form PACCs

Polyploid programs provide increased fitness both on an evolutionary timescale (across generations of organisms) and within the life span of a single organism. While the evolutionary programs can be acute responses to environmental anomalies or protection against mutation, such developmental programs are the norm for some specialized tissues. The evolutionary role precedes the developmental role of these programs as they arose in prokaryotes and single-celled eukaryotes long before the evolution of multicellular organisms. Cancer cells, as single-celled organisms and as decendants of a multicellular host, may engage either (or both) the evolutionary or developmental advantages of polyploidization. Understanding and applying these evolutionary and developmental programs may reveal the key stimuli that acutely and transiently induce polyploidy as well as reveal the benefits and costs of maintaining polyploid cells in cancer.



Hallmarks of Cancer

- self-sufficiency in growth signals
- insensitivity to antigrowth signals
- avoidance of apoptosis
- limitless replicative potential
- sustained angiogenesis
- tissue invasion and metastasis
- avoiding immune destruction
- deregulating cellular energetics

Enabled by:

- genome instability and mutation
- tumor promoting inflammation

Hallmarks of Lethal Cancer

- therapeutic resistance

Enabled by:

- polyploidization
- reversible cell cycle arrest

Fig. 1. The ability to access polyploid programs enables therapeutic resistance, the hallmark of lethal cancer. The PACC theory of cancer recurrence accounts for the ubiquity of recurrence in three steps. First, a few tumor cells respond to stress, e.g., chemotherapy or metabolic stress as a consequence of uncontrolled growth, through a polyploid program and form PACCs. Second, as part of cell enlargement and the polyploid program, PACCs pause proliferation, allowing adaptation to toxic environments while protecting DNA. This state is also associated with motility, which further enables metastasis. Third, when the stimulus is a therapy and is removed, the PACC can undergo depolyploidization, reinitiating tumor cell proliferation and recurrence.

PACCs form in response to many different natural and synthetic environmental stimuli such as hypoxia, lack of nutrients, changes in pH, or chemotherapy (5–31, 38). Within a multicellular organism, somatic cells that demonstrate chromosomal anomalies are commonly shunted to apoptosis. Moreover, somatic cells that experience severe environmental deviations, such as abnormally low pH, are lysed. In contrast, PACCs exhibit a protected cellular phenotype that is largely indifferent to a changing environment,

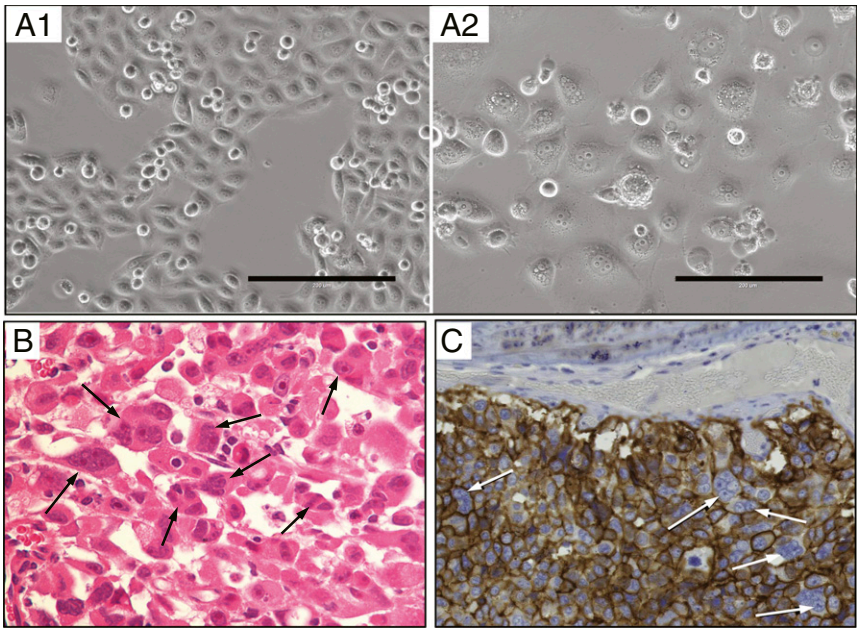


Fig. 2. Polyaneuploid cancer cells (PACCs). PACCs are observed in many cell lines. Prostate cancer cell line PC3 as an example before (*A.1*) and at 72 h after treatment with 10 nM docetaxel (*A.2*). They are also found in animal models (*B*: PC3 xenograft, 200,000 PC3 cells were injected s.c. in an NSG mouse; tumors were harvested on day 21 and processed for H&E [PACCs circled]) and in patients (*C*: lung metastasis from a patient with castrate-resistant prostate cancer stained with EpCAM).

whether local (changing tumor microenvironment) or systemic (toxic therapy). This type of resilience to environmental disruption through polyploidy and cell enlargement has been noted throughout evolution from unicellular to multicellular organisms. If PACCs can access such an evolutionarily defined response program, their ability to survive therapeutic stress may simply be a byproduct of this ancestral capacity (98). These evolutionary response programs of increasing cell size and DNA material are reflected in the developmental programs that are accessed by normal cells in response to stress. Once cancer cells mobilize this ability, they also gain the ability to survive and react to environmental stresses within the tumor microenvironment such as changes in oxygen, nutrients, and pH, i.e., the cancer swamp (99–101).

The formation of polyploid cells is observed across unicellular and multicellular eukaryotic organisms and is associated with distinct survival advantages (Table 1). Bacteria, protists (unicellular eukaryotic organisms), plants, fungi, invertebrate animals, and vertebrates demonstrate cell enlargement by polyploidy during development as well as temporal crises. Cells become polyploid through either cell fusion or more commonly by DNA duplication (102–106). DNA duplication occurs by alterations to the normal cell cycle through either mitotic slippage, endocycling (also termed endoreduplication or endoreplication), or endomitosis (cytokinesis failure) (105, 107–109). Mitotic slippage is a semicomplete cell cycle in which the cell fails to resolve the mitotic spindle assembly checkpoint and, after a delay, exits a prolonged mitosis with a 4N nucleus (110). Endocycling encompasses alternating gap and S-phases that can result in polyploid cells of varying ploidy, up to 1,000 copies of the genome (111). Many endocycling cells do not display early mitotic markers, such as nuclear envelope breakdown, and some exhibit truncated S-phases where late-replicating DNA in heterochromatic regions is not fully duplicated, leading to genomic deletions (76). Endomitosis is achieved by bypassing cytokinesis or late mitosis, resulting in multinucleate cells or lobulated nuclei when anaphase is not completed (e.g., the formation of megakaryocytes). The mitotic cycle and the endocycle that results in replication of the genome without complete mitosis are linked through tight regulation of the cell cycle program at multiple checkpoints (81, 104, 112–120). The failure of nuclear division, or endomitosis, can also be achieved in two ways. If only cytokinesis is blocked, multinucleated polyploid cells are formed, but if karyokinesis is inhibited, polyploid cells possess a single nucleus.

Traditionally, in the field of evolutionary biology, the preservation and occurrence of polyploidy preserved across the tree of life have been attributed to the idea that WGD contributes to genome stability. The increased DNA content may protect the cell and the organism from mutations or chromosomal aberrations as a result of DNA damage (121–123) (Table 1). If left unpurged or unrepaired, the inexorable accumulation of deleterious genetic events has been termed Muller's ratchet. Indirect evidence for this argument can be found in the many redundant paralogs of multiple genes and pathways observed in the genomes of higher organisms (75, 123–125). Essentially, the presence of an extra copy of chromosomes serves as a genetic backup system to protect against the effects of mutations and DNA strand breaks that would otherwise cause a cell to undergo apoptosis and be lost from the tissue pool (126). WGD has also been proposed as a mechanism to increase genome instability to allow organisms to generate more heritable variation in response to environmental stress (74, 127–130). The increase in DNA material also correlates

Table 1. Postulated consequences for polyploidization including whole-genome doubling

Consequence	Description
Genomics	
1. Increased genomic stability	Extra copies of genes allow organisms to avoid lethal genomic damage, e.g., preventing Muller's ratchet in protists.
2. Increased heritable variation	The increased genomic material allows increased mutation in response to stress. Genetic instability creates progeny of various fitness allowing selection of a robust clone, e.g., antibiotic resistance in some yeast strains.
3. Self-genetic modification	Increased genomic material provides self-genetic modification through directed reprogramming, e.g., antibiotic resistance in some bacteria strains.
4. New functionality	Redundant genomic material allows mutation to achieve a new functionality. For example, two pairs of limbs allow one pair to become wings.
Function	
5. Induction of quiescence	Halting of the cell cycle leads to a nonproliferative state as a mechanism to protect the nondividing genome while stress is present, e.g., *Entamoeba histolytica*.
6. Increased storage capacity	Increased cell size increases storage capacity needed for sustained quiescence (genomic material is a passenger), e.g., plant vacuoles.
7. Increased cell function	Increased cell size increases cell function (genomic material is a passenger), e.g., osteoclast fusion for the production of acid to lyse bone.
8. Increased metabolic capacity	Increased gene dosage increases production of RNA and protein products necessary for increased cell metabolism for growth, e.g., megakaryocytes.
9. Increased toxin protection	Increased gene dosage increases production of RNA and protein products necessary to protect from oxidative damage and cell size may protect from short-term environmental toxic stresses, e.g., hepatocytes.

with increased cell size, suggesting that WGD may be a strategy for growth of either the cell, the tissue, or the organism.

The diverse functional roles of polyploidy across evolution and development testify to the scope of cell enlargement reaching beyond genome size or stability (121, 122, 124). Indeed, cell enlargement may allow a cell to perform more functions independently, as opposed to adhering to and dividing labor among multiple cells. Greater functionality and independence can be achieved only by polyploidization. Cell enlargement provides, for example, increased amounts of biomolecules to be utilized for nutrients, for increased organelle function, or to mitigate the effects of environmental change. Endoreplication efficiently provides the nutrients and materials needed to support developing eggs or embryos, such as *Drosophila* follicle and nurse cells and mammalian trophoblasts (31). The biomass of polyploid cells in fruit fly larvae provides nutrients during larval feeding (31) and plants use increased cell size to increase nutrient storage (86). Cell enlargement for enhancing organelle functions results from increased gene dosage and subsequent downstream machinery for the increased production of cellular building blocks, including RNA, proteins, and lipids as well as increased energy production though increased numbers of mitochondria (88, 92). Polyploidization provides an economy of scale for cell metabolic processes.

While genetic programming also relates to long-term adaptions, cells adapt to oxidative stress in the short term by metabolic reprograming (80). In mammals, increased machinery for platelet production is facilitated by megakaryocytes that increase intracellular materials and metabolism by serially doubling their genomes and inhibiting cytokinesis (76, 78, 131). Finally, cell enlargement that mitigates the effects of environmental change is observed in, for example, protists and plants. One group of protists, the Foraminifera, toggle between haploidy, diploidy, and polyploidy during harsh and toxic conditions in oceans, lakes, and soil (132–134). During temporary decreases of light or water, polyploid leaf and root cells maximize surface area via endoreplication to maximize surface area and uptake (135). Plant seedlings also utilize polyploidy for fast growth out of the dark soil and into the light. During injury, salamander cells and mammalian liver cells can regenerate functional tissue by accessing polyploid programs, even when cell division is blocked (76). It is noteworthy that polyploid cells facilitate development and survival during harsh conditions not only by their increased genomic material, but by their increased cellular contents and sheer size (127, 128).

Benefits to Polyaneuploidy for Tumor Survival: Predictions

The generation of WGD and consequent polyploidy and aneuploidy are well documented in the cancer literature (67, 136). PACCs as a cancer cell life history state can have multiple nonmutually exclusive advantages that act in a cooperative manner across the cancer cell population within a tumor (137, 138). This polyploidization appears to be a necessary step to induce a pause in the cell cycle to protect the cell from stress-induced DNA damage. Since loss of contact inhibition and concomitant uncontrolled proliferation is a hallmark of cancer, accessing a polyploid program after DNA replication may be the only mechanism available for cancer cells to exit the cell cycle (65). We predict that 2N+ cancer cells do not exit the cell cycle to a nonproliferative state unless they become PACCs. Further work will define the interrelationships between PACCs, quiescence, senescence, and cell cycle control.

Increased genomic material, such as is observed in PACCs, has been noted in evolutionary and developmental biology. Being a 4N+ cell provides several potential advantages, including increased genetic stability to prevent apoptosis in a cell with damaged DNA as well as the generation of beneficial mutations to promote survival of resistant progeny (Table 1) (76, 79, 139). PACCs eventually undergo depolyploidization to produce "typical" non-PACC progeny and repopulate tumor sites through cell division, amitotic mechanisms (e.g., neosis), or both (16, 34, 37, 91, 92, 140, 141). The timing of PACC depolyploidization is relevant to understanding cancer recurrence through the generation of proliferative progeny as well as the exit from apparent "dormancy" (or paused proliferation) often associated with cancer metastasis (142). Understanding the dynamics of polyploidization, quiescence of PACCs, and depolyploidization may also shed light on how these structurally abnormal cells evade recognition and subsequent destruction by the immune system (22, 25, 61). In our own experiments, when PACCs generate progeny, we do not observe generation of multiple nonviable cells as would be suggested by a genetic instability mechanism (29, 99). Their 2N+ progeny have increased resistance to different forms of stress. We predict that PACCs do not utilize genetic instability as the mechanism to generate progeny with therapeutic resistance. Further work will define the role of genome protection through quiescence versus mutation in the generation of progeny and population rescue of tumors.

The increased cell size of PACCs is not in dispute, but the functional purpose for this increase in cellular machinery has not been established (Table 1) (8, 16, 20, 23, 34, 35, 143). Evolutionary and developmental polyploidization programs utilize the additional genomic material to provide the building blocks for increased cell metabolism. It is possible that the increased cell size allows PACCs to store more energy molecules (e.g., lipids, proteins, carbohydrates) and to survive extended periods of dormancy. It is also possible that increased cell size (with its concomitant decreased surface-to-volume ratio) provides protection from toxin and oxidative stress via increased production of RNA and protein for protective pathways. We predict that PACCs have altered metabolism as compared to 2N+ cancer cells that is shunted toward cell survival and detoxification while downregulating cell proliferation pathways. Further work will characterize the mechanisms that contribute to PACC survival under stress as well as the mechanisms that release PACCs from quiescence and reenter the cell cycle to begin proliferation.

Conclusions

We present a unifying theory to explain cancer recurrence and lethality. The hallmarks of cancer provide the framework for tumorigenesis and are complemented by the hallmark of therapeutic resistance that is enabled by polyaneuploidy that enables recurrence and lethality (39). Specifically, therapeutic resistance enabled by the access of a polyploidization program represents an additional hallmark of cancer, representing a "hallmark of lethal cancer." Access to these evolutionary and developmental programs allows a paused cell cycle and concomitant WGD, further enabling subsequent cellular capabilities of sustained dormancy and genetic modification that result in resistant progeny and tumor regrowth.

Data Availability. There are no data underlying this work.

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ORIGINAL PAPER

High KIFC1 expression is associated with poor prognosis in prostate cancer

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Abstract

Kinesins play important roles in the progression and development of cancer. Kinesin family member C1 (KIFC1), a minus end-directed motor protein, is a novel Kinesin involved in the clustering of excess centrosomes found in cancer cells. Recently KIFC1 has shown to play a role in the progression of many different cancers, however, the involvement of KIFC1 in the progression of prostate cancer (PCa) is still not well understood. This study investigated the expression and clinical significance of KIFC1 in PCa by utilizing multiple publicly available datasets to analyze KIFC1 expression in patient samples. High KIFC1 expression was found to be associated with high Gleason score, high tumor stage, metastatic lesions, high ploidy levels, and lower recurrence-free survival. These results reveal that high KIFC1 levels are associated with a poor prognosis for PCa patients and could act as a prognostic indicator for PCa patients as well.

Keywords Prostate cancer · KIFC1 · Centrosome clustering · Ploidy · Kinesins · Poly-aneuploid cancer cells

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer (aside from non-melanoma skin cancer) in men worldwide and the second leading cause of cancer deaths in American men [8]. In 2018 alone there were over 1.2 million new cases of PCa and over 300,000 deaths from PCa in the world [8]. The incidence rates and mortality rates of PCa are strongly correlated with age, with the highest incidence rates being in elderly men above 65 years of age [9]. While localized disease has a 5-year survival rate of 100%, patients that progress to metastatic disease have a five-year survival rate of only 30% [10]. Despite many therapeutic advances, metastatic prostate cancer remains incurable.

When stressed, cancer cells can undergo endoreplication, failed cytokinesis, or fusion which generates excess centrosomes within the cell. These same mechanisms also generate higher levels of genomic content in cancer cells taking cancer cells from aneuploid to polyploid or poly-aneuploid [11]. It is currently hypothesized that cells with elevated genomic content, e.g., polyploid giant cancer cells or poly-aneuploid cancer cells, may have advantages for surviving in stressful environments [12–18]. In animal cells there is a centrosome located at each spindle pole and each centrosome will be delivered to a single daughter cell during cell division and then eventually duplicate in the daughter cells S phase [19]. The correct distribution of centrosome number is critical for normal functioning of centrosome signaling and normal cell division [20]. In healthy mammalian cells, centrosome regulation is strictly maintained, however, centrosome aberrations are commonly observed in tumors [19]. Centrosome amplification can predispose cells to chromosomal instability (CIN) [21] and can also generate a multi-polar instead of a bi-polar spindle [22]. Multipolarity is normally lethal but cells are able to avoid death by clustering their centrosomes and inducing bi-polar division.

Centrosome clustering in cancer cells is mediated by Kinesin family member C1 (KIFC1) (common paralogs: HSET, KIFC3, KIFC2, KIF14). KIFC1 is a minus end-directed motor protein involved in spindle pole organization and formation [23] and the clustering of excess centrosomes found in cancer cells [24, 25]. KIFC1 allows cancer cells with multiple centrosomes to survive cell division. KIFC1 is

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non-essential for normal somatic cells but necessary for the proper division of cancer cells with excess centrosomes [26, 27]. High KIFC1 levels have been reported in various cancer types such as breast cancer [28], hepatocellular carcinoma [29], and ovarian cancer [30]. Recently, KIFC1 has shown to be an important factor in prostate cancer progression and drug resistance by inhibiting cell death and conferring docetaxel resistance [24].

In this study, we demonstrate that high KIFC1 expression is associated with high grade, high stage, high ploidy, and metastatic PCa. Additionally, high KIFC1 levels are associated with poor recurrence-free survival in PCa as well as other cancer types.

Methods

The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) Cell 2015 [1], TCGA-PRAD provisional (accessed Nov 20, 2019), German Cancer Center (DKFZ) Cancer Cell 2018 [2], and Memorial Sloan Kettering Cancer Center (MSKCC) Cancer Cell 2010 [3] datasets were accessed via cBioPortal [4, 5]. The TCGA-PRAD provisional dataset was also accessed using UCSC Xena browser [6]. The TCGA-PRAD provisional dataset was used to analyze Gleason score and tumor stage, and compare PCa primary tissue with matched solid (normal) tissue. The TCGA-PRAD, Cell 2015 [1] dataset was used to analyze absolute doubling events and absolute extract ploidy. The DKFZ [2] dataset was used to analyze Gleason score and tumor stage. The MSKCC [3] dataset was used to analyze KIFC1 expression in primary and metastatic PCa and Gleason score. The MSKCC [3] dataset was also used to assess high/low KIFC1 expression and recurrence-free survival. GEPIA [7] was used to generate Kaplan Meier curves of KIFC1 expression and recurrence-free survival in TCGA-PRAD as well as thirty-three different TCGA datasets of different cancer types.

Cell culture

PC3 and DU145 cells obtained from ATCC (PC3 cells were modified at the University of Michigan), were cultured in RPMI medium plus 10% FBS (VWR, product number: 97068–085) and 1% penicillin streptomycin. All cells were cultured in an incubator at 37 °C with 5% CO2. All cells underwent regular mycoplasma testing and STR profiling. Poly-aneuploid cancer cells (PACCs) were generated by treating PC3 or DU145 cells with 5 nM docetaxel for 72 h. PACCs were then isolated from the mixed population by filtration (pluriStrainer 15 µm cell strainer, SKU 43–50,015-03).

Imaging

Phase-contrast images were taken of control PC3 cells and PC3 PACCs on an EVOS M7000 imaging system.

Flow cytometry

Control PC3 cells and PC3 PACCs were stained with FxCycle PI/RNase Staining Solution (ThermoFisher Scientific cat# F10797). Both samples were run on a Bio-Rad S3E cell sorter and analyzed using FlowJo.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with Halt Protease and Phosphatase Single-Use Inhibitor cocktail (100x) (REF 78,442). Protein was fractionated on a 4–20% TGX gel and transferred to a nitrocellulose membrane. Membranes were incubated with anti-KIFC1 antibody (Abcam, Cat# ab172620, RRID: AB_2827938; 1:10,000) and anti-ß-Actin antibody (Sigma-Aldrich Cat# A5441, RRID: AB_476744; 1:10,000). Anti-rabbit and anti-mouse secondary were used for detection on a LI-COR Odyssey.

Densitometry

Fiji software was used for densitometric analysis of the western blots. Resulting graphs are representative of three separate western blots.

Statistical analysis

Statistical analysis was performed using GraphPad's Prism software (GraphPad Software, San Diego, CA). One-way ANOVA or unpaired t-tests were performed to determine P values and significance across or between samples.

Results

High KIFC1 levels are associated with higher Gleason score

KIFC1 mRNA expression was assessed in PCa patients with various Gleason scores. KIFC1 mRNA expression was increased with higher Gleason scores in three separate PCa datasets (Fig. 1). Both the TCGA-PRAD provisional (accessed November 20, 2019) and DKFZ [2] datasets demonstrated KIFC1 mRNA expression significantly increasing with higher Gleason score ($P < 0.0001$)

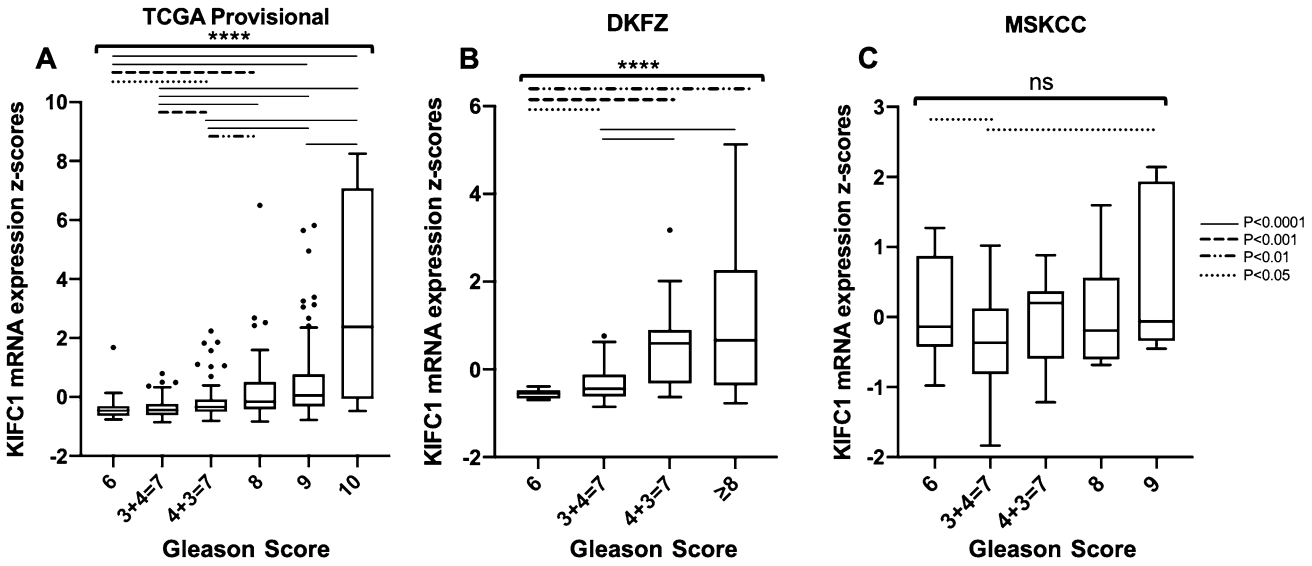

Fig. 1 KIFC1 mRNA expression and Gleason score. **a** TCGA-PRAD provisional data (accessed on November 20, 2019) of KIFC1 mRNA expression and Gleason scores. (Gleason score: 6, $n=45$; $3+4=7$, $n=153$; $4+3=7$, $n=101$; 8, $n=57$; 9, $n=138$; 10, $n=4$). **b** DKFZ data of KIFC1 mRNA expression and Gleason scores (Gleason score: 6, $n=13$; $3+4=7$, $n=69$; $4+3=7$, $n=18$; ≥ 8, $n=18$). **c** MSKCC data of KIFC1 mRNA expression and Gleason scores (Gleason score: 6, $n=41$; $3+4=7$, $n=53$; $4+3=7$, $n=23$; 8, $n=10$; 9, $n=11$. P values determined by one-way ANOVA or unpaired t-test. ns, $P>0.05$; ****$P\leq 0.0001$

(Fig. 1a, b). The MSKCC [3] dataset demonstrated a trend of KIFC1 mRNA expression increasing with higher Gleason score ($P=0.0584$) (Fig. 1c). Patients with higher Gleason scores had higher KIFC1 mRNA expression indicating these patient samples most likely had higher levels of centrosome clustering mediated by KIFC1.

KIFC1 expression may increase with PCa stage

Across the 393 patient samples assessed in the TCGA-PRAD provisional dataset (accessed November 20, 2019) KIFC1 mRNA expression was significantly increased with higher tumor stage ($P<0.0001$) (Fig. 2a). Similar trends were shown across the 116 patient samples

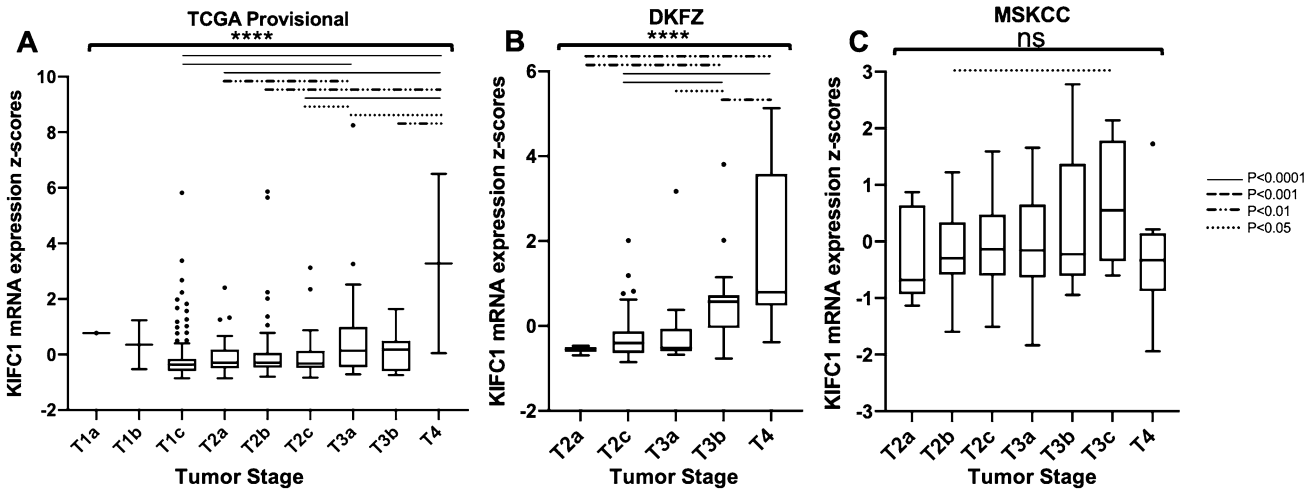

Fig. 2 KIFC1 mRNA expression and Tumor Stage. **a** TCGA-PRAD provisional (accessed on November 20, 2019) data of KIFC1 expression and tumor stage (T1a, $n=1$; T1b, $n=2$; T1c, $n=174$; T2a, $n=56$; T2b, $n=55$; T2c, $n=50$; T3a, $n=36$; T3b, $n=17$; T4, $n=2$). **b** DKFZ data of KIFC1 expression and tumor stage (T2a, $n=8$; T2c, $n=66$; T3a, $n=16$; T3b, $n=19$; T4, $n=7$). **c** MSKCC data of KIFC1 expression and tumor stage (T2a, $n=9$; T2b, $n=48$: T2c, $n=29$; T3a, $n=30$; T3b, $n=13$; T3c, $n=4$; T4, $n=8$). P values determined by one-way ANOVA or unpaired t-test. ns, $P>0.05$; ****$P\leq 0.0001$

in the DKFZ [2] dataset where KIFC1 mRNA expression was also significantly increased with higher tumor stage ($P < 0.0001$) (Fig. 2b). Data from the 141 patient samples in the MSKCC [3] dataset demonstrated KIFC1 expression was highest in tumor stage T3C but did not significantly increase with higher tumor stage ($P = 0.2879$) (Fig. 2c). KIFC1 mRNA expression increased as tumor stage increased in multiple datasets indicating higher stage tumors likely have higher levels of KIFC1 mediated centrosome clustering.

KIFC1 expression is highest in metastatic PCa

KIFC1 mRNA expression was higher in metastatic PCa lesions than in the primary tumors. In TCGA-PRAD provisional dataset primary tumor KIFC1 mRNA expression was higher than matched solid tissue (Fig. 3a). The MSKCC [3] dataset showed KIFC1 mRNA expression was higher in metastatic lesions than in the primary tumor (Fig. 3b). KIFC1 expression was higher in primary tumors than in matched solid (normal) tissue as well as higher in metastatic lesions than in the primary tumor indicating that metastatic PCa lesions may require higher levels of centrosome clustering mediated by KIFC1.

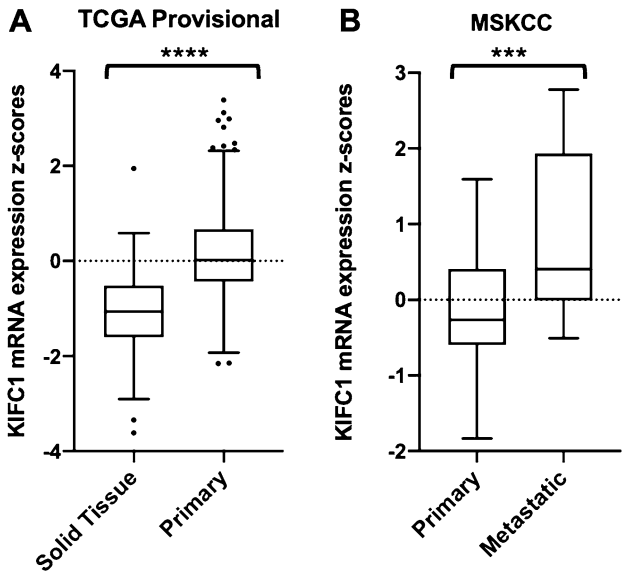

Fig. 3 KIFC1 mRNA expression and metastatic disease. **a** TCGA-PRAD provisional data comparing KIFC1 mRNA expression in matched solid (normal) tissue ($n = 52$) and primary PCa ($n = 479$). **b** MSKCC data comparing primary ($n = 130$) and metastatic PCa ($n = 9$) to KIFC1 mRNA expression. *P* values determined by unpaired t-test. ***$P \leq 0.001$; ****$P \leq 0.0001$

High KIFC1 expression is associated with lower recurrence-free survival in PCa as well as other cancer types

This study compared recurrence-free survival between patients with high and low KIFC1 mRNA expression levels. Recurrence-free survival in PCa describes time to biochemical recurrence (BCR) which refers to a rise in the blood level of prostate-specific antigen (PSA) in PCa patients after initial treatment. In the MSKCC [3] PCa dataset patients with higher than median KIFC1 mRNA expression levels had lower recurrence-free survival ($P = 0.0207$) (screenshots taken directly from bestasatsis.com) (Fig. 4a).

We also used GEPIA [7] to generate Kaplan Meier curves using data from TCGA-PRAD dataset. TCGA-PRAD patients with above-median KIFC1 expression levels had lower recurrence-free survival compared to patients with below median KIFC1 expression ($P = 4.2E\text{-}05$) (Fig. 4b). Next, we looked at KIFC1 expression across multiple cancer types. Thirty-three TCGA datasets available on GEPIA [7] (including TCGA-PRAD) were analyzed for KIFC1 expression. Patients with above-median KIFC1 expression had lower recurrence-free survival when assessing all of the cancers at once (Fig. 4c) ($P = 6.4E\text{-}15$). Additionally, when analyzed separately, eleven of the thirty-three datasets analyzed had significant differences ($P < 0.05$) when comparing median high to low KIFC1 mRNA expression: adrenocortical carcinoma (ACC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), mesothelioma (MESO), prostate adenocarcinoma (PRAD), sarcoma (SARC), thyroid carcinoma (THCA), and uveal melanoma (UVM). Overall, high KIFC1 mRNA expression was associated with lower recurrence-free survival in PCa as well as other cancer types, indicating that patients with these cancer types and high KIFC1 levels have a poorer prognosis.

High KIFC1 expression is associated with higher ploidy levels

In TCGA-PRAD, Cell 2015 [1], dataset KIFC1 mRNA expression was analyzed for absolute genome doublings and absolute extract ploidy. Absolute genome doublings and absolute extract ploidy were determined using the ABSOLUTE algorithm [31] which deduces malignant cell ploidy directly from analysis of somatic DNA alterations. KIFC1 mRNA expression was significantly increased in samples with absolute genome doublings ($P < 0.0001$) (Fig. 5a). KIFC1 expression was also significantly higher in samples with higher absolute extract ploidy (Fig. 5b) ($P < 0.0001$).

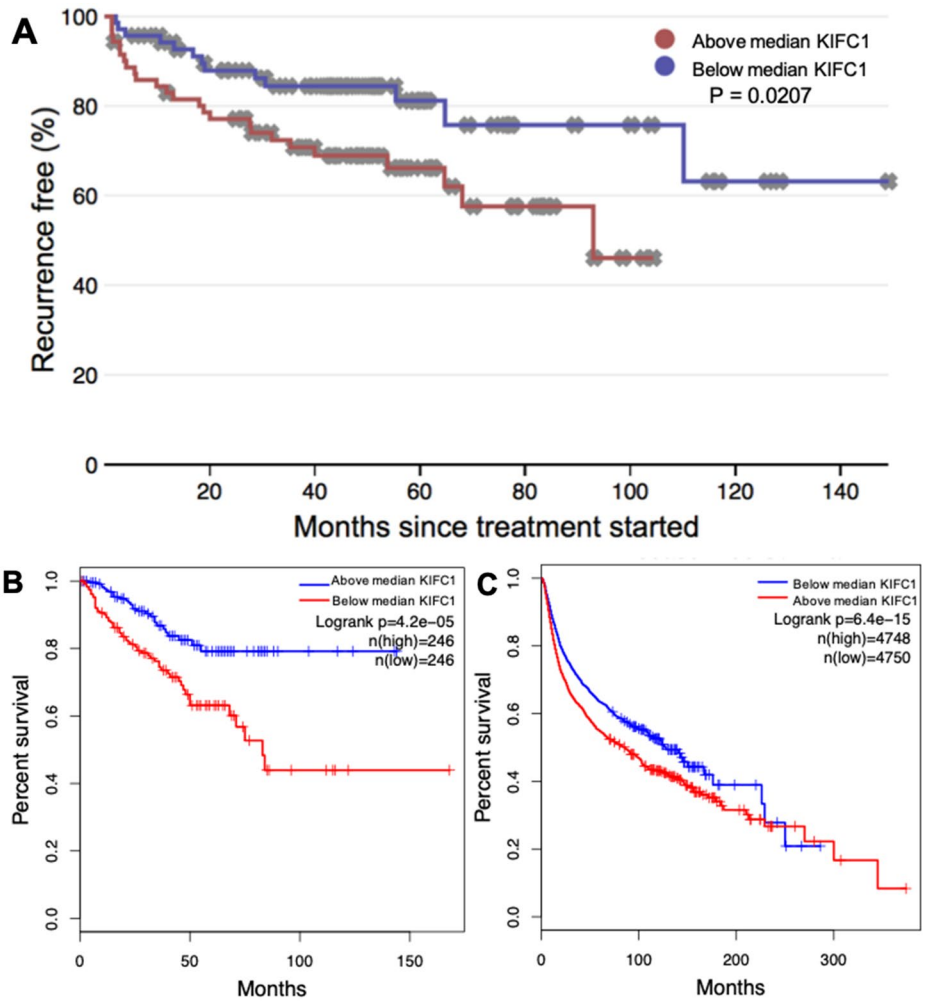

Fig. 4 High KIFC1 expression is correlated with lower recurrence-free survival in PCa and other cancer types. Kaplan Meier curves and P values generated from betastasis.com and GEPIA comparing KIFC1 expression and recurrence-free survival. **a** Kaplan Meier curve from betastasis.com utilizing the MSKCC dataset comparing above/below median KIFC1 mRNA expression in PCa (*n*(above median) = 70; *n*(below median) = 71) (*P* = 0.0207). **b** Kaplan Meier curve generated from GEPIA of the TCGA-PRAD dataset comparing above/below median KIFC1 expression (*n*(above median) = 246; *n*(below median) = 246) (*P* = 4.2E-05). **c** Kaplan Meier curve generated from GEPIA of the thirty-three compiled TCGA datasets available comparing above/below median KIFC1 expression and recurrence-free survival across multiple cancer types (*n*(above median) = 4748; *n*(below median) = 4750) (*P* = 6.4e-15)

To directly assess ploidy in cancer cells, we generated and isolated poly-aneuploid cancer cells (PACCs) from two different cell lines. PACCs are cancer cells containing a doubled (or greater multiple, e.g., 4 N, 8 N…) aneuploid genome, also referred to as polyploid giant cancer cells (PGCCs) or multi-nucleated cancer cells [11–18]. PACCs were generated by treating cells with 5 nM docetaxel for 72 h. They were then isolated from the population using a 15um filter. PACCs were visualized by phase-contrast imaging (Fig. 5c). Additionally, PACC ploidy was verified by the assessment of DNA content in all cells by flow cytometry (Fig. 5d). Utilizing western blotting we demonstrated that KIFC1 protein levels were higher in PACCs than their parental cell lines (Fig. 5e). We also demonstrated this by quantifying three separate western blots via densitometric analysis (Fig. 5f). This data demonstrated higher ploidy samples are associated with high KIFC1 mRNA expression and high KIFC1 protein levels. This could indicate that higher ploidy cancers need higher levels of centrosome clustering mediated by KIFC1.

Discussion

Genomic instability, a hallmark of cancer, facilitates the acquisition of mutations that can drive cancer evolution and drug resistance. Aneuploidy, the presence of an abnormal number of chromosomes in a cell, is a characteristic of genomic instability and introduces more genetic variation, allowing cells to adapt in changing or stressful environments [21]. Aneuploid cells that undergo endoreplication, failed cytokinesis, or fusion become polyploid/poly-aneuploid cancer cells (cells containing more than two paired sets of chromosomes) and contain excess centrosomes. Excess centrosomes can lead to cell death when a cell attempts multipolar division. However, centrosomes can be clustered to promote bi-polar division and cell survival. KIFC1 is an important kinesin for cancer cells that clusters excess centrosomes to allow for the successful division and survival of cancer cells. KIFC1, while not expressed in somatic cells, is widely expressed in cancers such as ovarian [30], breast [28], bladder [32], lung [33], kidney [34], and we report here, prostate. Elevated levels of KIFC1 have been shown

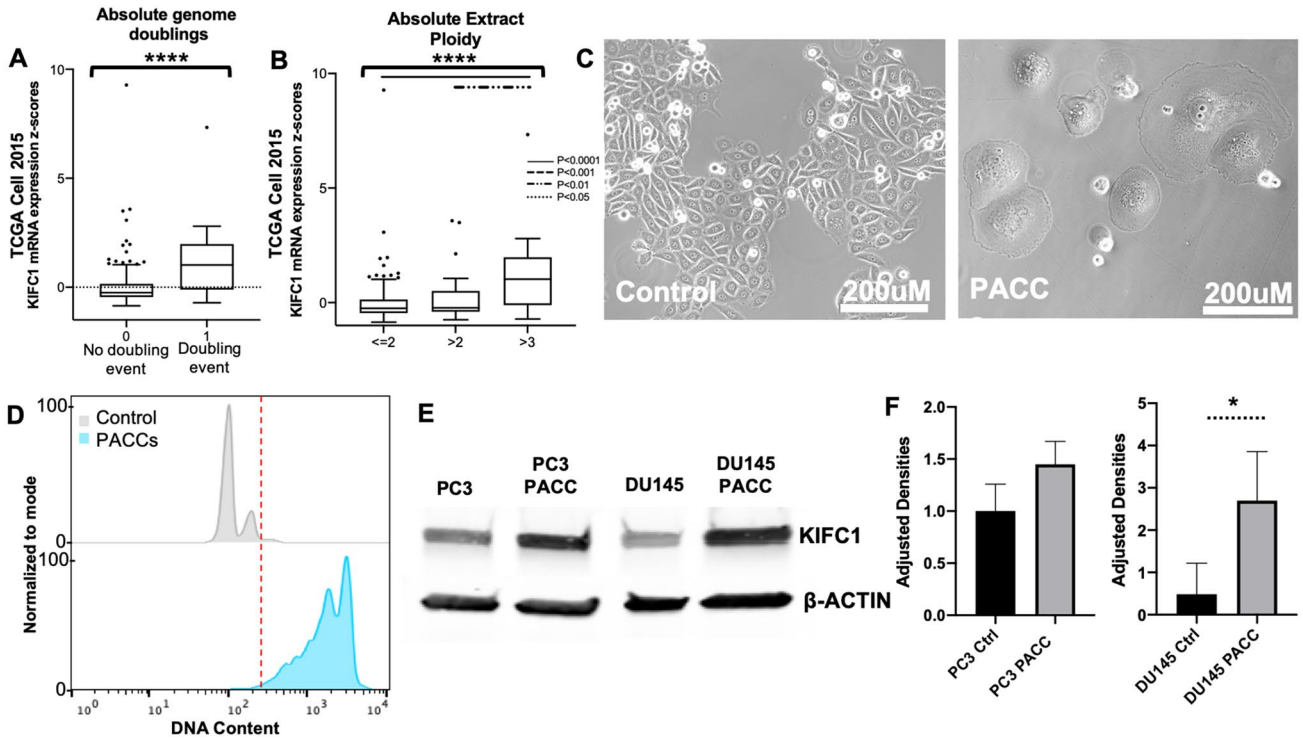

Fig. 5 Relationship of KIFC1 expression and ploidy levels. **a** TCGA-PRAD Cell 2015 data comparing KIFC1 mRNA expression and absolute genome doublings (Without a doubled genome, $n=229$; With a doubled genome, $n=18$). **b** TCGA-PRAD Cell 2015 data comparing KIFC1 mRNA expression and absolute extract ploidy ($<=2$, $n=192$; >2, $n=37$; >3, $n=18$). **c** Phase-contrast images of control PC3 cells and PC3 PACCs. **d** Flow cytometry analysis of DNA content in control PC3 cells and PC3 PACCs after filtration enrichment (all cells to the right of the red line are > 4N). **e** Western blot comparing KIFC1 expression in PC3 cells, PC3 PACCs, DU145 cells, and DU145 PACCs (results are representative of three separate experiments). **f** Densitometric analysis of western blots. *P* values determined by one-way ANOVA or unpaired t-test. $*P \leq 0.05$; $****P \leq 0.0001$

to confer drug resistance in breast cancer [35] as well as prostate cancer [24]. KIFC1 has recently been described as important to PCa progression [24].

The Gleason scoring system and the TNM staging system are utilized to categorize prostate cancer aggressiveness and disease extent. The Gleason scoring system is one of the most common grading systems used for PCa [10] and Gleason scores have been shown to be powerful indicators of PCa recurrence and mortality [36]. We report here that patients with higher Gleason scores have higher levels of KIFC1 mRNA expression suggesting the capacity for higher levels of KIFC1 mediated centrosome clustering in those patients' tumors. The DKFZ [2] and TCGA-PRAD provisional (accessed Nov. 20, 2019) datasets demonstrated significant increases in KIFC1 mRNA expression with higher T stages in primary tumors. While the MSKCC dataset did not show significant differences, it did show a trend of increasing KIFC1 mRNA expression and higher T stages and Gleason scores. These findings that KIFC1 mRNA expression increases as tumor stage progresses in multiple datasets indicates that higher stage tumors may require higher levels of KIFC1 mediated centrosome clustering.

Further investigation of KIFC1 mRNA expression in clinical datasets led to the evaluation of KIFC1 mRNA expression in metastatic lesions. In this study, KIFC1 mRNA expression was found to be higher in metastatic PCa lesions compared to the primary tumor. It was also shown that higher KIFC1 levels are present in primary tumor samples compared to matched solid (normal) tissue samples. These results reveal that higher levels of centrosome clustering, mediated by KIFC1, are present in metastatic lesions compared to the primary tumor or normal tissue.

KIFC1 expression not only increased with higher Gleason scores, tumor stages, and metastatic disease; high KIFC1 expression was also associated with lower recurrence-free survival in PCa patients as well as patients with other cancer types. A significant difference was shown between the high/low KIFC1 expression groups in PCa. Additionally, thirty-three TCGA datasets of other cancer types, when analyzed simultaneously, demonstrated a trend with higher KIFC1 expression and lower recurrence-free survival. This data delineates KIFC1 as a potential prognostic factor in PCa and also demonstrates that high KIFC1 mRNA expression

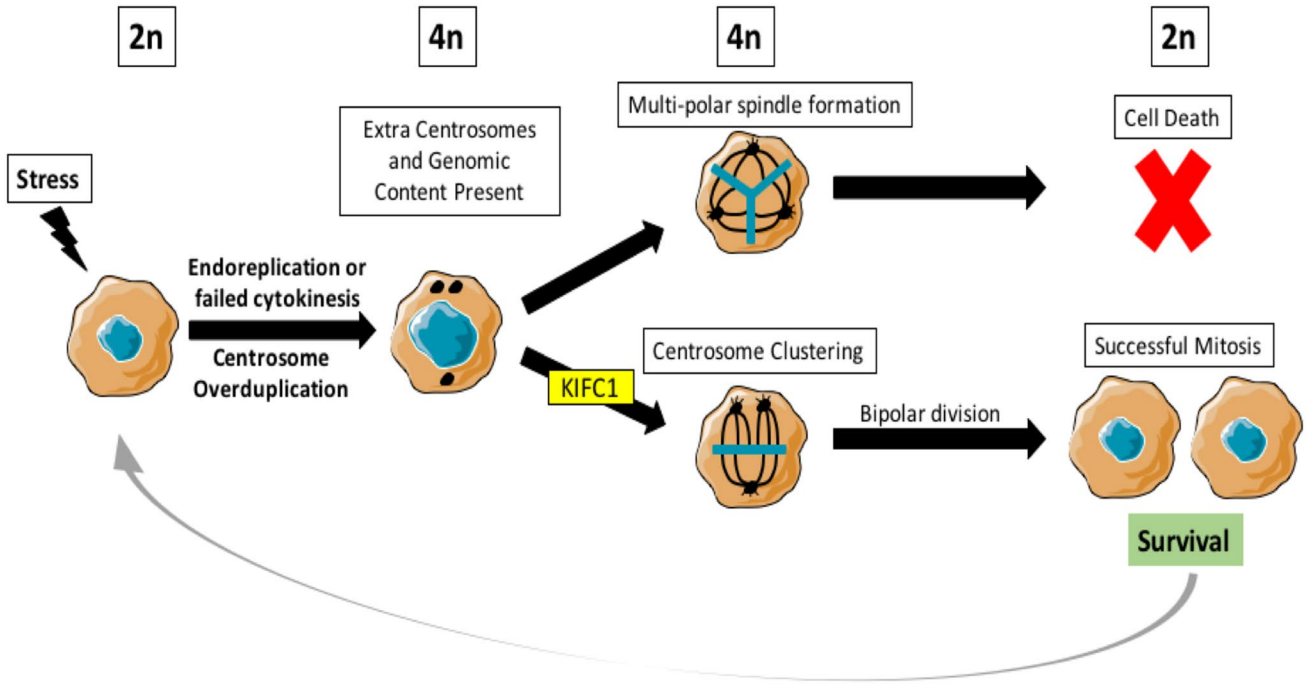

Fig. 6 KIFC1 allows cancer cell survival through centrosome clustering. When stressed, cells may undergo endoreplication, failed cytokinesis, or fusion causing an over duplication of centrosomes and an increase in genomic content. Cells with over-duplicated centrosomes may attempt to divide unevenly and form a multipolar spindle. KIFC1 is capable of taking cells with multipolar spindles and clustering their centrosomes allowing for bipolar division. This bipolar division allows cell survival and continued proliferation

is not only associated with a poor prognosis in PCa, but in many other cancer types as well.

KIFC1 mRNA expression was higher in samples with absolute genome doublings and higher absolute extract ploidy. In addition to KIFC1 mRNA expression, this study also observed KIFC1 protein levels in higher ploidy cells (PACCs). PACCs are large atypical cancer cells with multiple copies of DNA [15]. PACCs have been documented in several different baseline cancer lines including ovarian cancer cell lines HEY and SKOv3, and breast cancer cell line MDA-MB-231 [15]. Further investigation of PACCs showed that KIFC1 protein levels were higher in PCa PC3 PACCs and PCa DU145 PACCs compared to control PC3 and DU145 cells. Overall, as ploidy levels increased so did KIFC1 levels demonstrating that higher ploidy cells contain higher levels of KIFC1.

Our study promotes a model for KIFC1 as a novel kinesin in PACC and other high ploidy cell survival. Cancer cells that undergo endoreplication, failed cytokinesis, or fusion contain over-duplicated centrosomes and higher levels of genomic content. During division, a multi-polar spindle can form in response to the presence of excess centrosomes. Microtubules then bind incorrectly and genomic content is distributed unevenly between the multiple poles. This ultimately results in either (1) A cell not progressing past the mitotic checkpoint or (2) Death from the inability to transfer the necessary amount of genomic material to each daughter cell. KIFC1 is a novel kinesin that clusters excess centrosomes in cancer cells allowing the formation of bi-polar spindles, normal division, and cell survival (Fig. 6).

This study presents evidence of high KIFC1 expression in PCa indicating a poorer prognosis. High KIFC1 expression was correlated with high Gleason score, high tumor stage, metastatic lesions, lower recurrence-free survival, and higher ploidy levels. This is also the first evidence that showed PACCs had higher KIFC1 protein levels than normal parental cells. This information leads us to hypothesize if KIFC1 could be inhibited effectively in combination with other treatment options it would lead to increased cancer cell lethality.

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Data availability The data that supports the findings of this study are available in The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) Cell 2015 [1], TCGA-PRAD Provisional (accessed Nov 20, 2019), German Cancer Center (DKFZ) Cancer Cell 2018 [2], and Memorial Sloan Kettering Cancer Center (MSKCC) Cancer Cell 2010 [3] datasets. Data were derived from the following databases available in the public domain: cBioPortal [4, 5], UCSC Xena browser [6], betastasis.com, and GEPIA [7].

Code availability Not Applicable.

Declarations

Conflict of interest KJP discloses that he is a consultant to Cue Biopharma, Inc. as well as a founder and equity holder in Keystone Biopharma, Inc. No other authors have conflicts of interest to disclose.

Ethical approval Not Applicable.

Consent to participate Not Applicable.

Consent for publication All authors consent to the publication of this manuscript.

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