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Contributions of the distinct biophysical phenotype of polyploidal giant cancer cells to cancer progression

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ABSTRACT

Polyploid giant cancer cells (PGCCs) are a commonly observed histological feature of human tumors and are particularly prominent in late stage and drug resistant cancers. The chromosomal duplication conferred by their aneuploidy gives rise to DNA damage resistance and complex tumor cell karyotypes, a driving factor in chemotherapy resistance and disease relapse. Furthermore, PGCCs also exhibit key cytoskeletal features that give rise to a distinct biophysical phenotype, including increased density of polymerized actin and vimentin intermediate filaments, nuclear and cytoskeletal stiffening, increased traction force, and migratory persistence. Despite recent research highlighting the role PGCCs play in cancer progression, this population of tumor cells remains poorly characterized in terms of their biophysical properties. In this review, we will discuss the various aspects of their biomolecular phenotype, such as increased stemness as well as a mixed EMT signature. These features have been extensively associated with tumorigenesis and recurrence, and aggressive cancers. Additionally, we will also examine the distinct PGCC cytoskeletal features of actin and filamentous vimentin. Specifically, how the differential organization of these networks serve to support their increased size and drive migratory persistence. These findings could shed light on potential therapeutic strategies that allow for specific elimination or mitigation of the invasive potential of these polyploid cancer cells. Lastly, we will examine how the biophysical and molecular phenotype of PGCCs combine to tip the scale in favor of promoting cancer progression, presenting an important target in the clinical treatment of cancer.

1. Relevance of PGCCs in cancer progression

1.1. Clinical implications of polyploidy

In recent years, there has been an alarming rise in annual cancer incidence rates within the US, with an average of 1.7 million cases per annum and over 1.8 million projected cases in 2020 [1]. Despite advancements in treatment methodologies and adjuvant therapies, cancer remains one of the highest causes of death within the US [2]. High levels of intratumoral heterogeneity are a major challenge in the treatment of cancer [3–6]. Specifically, small subpopulations of chemoresistant and highly tumorigenic cells can resist initial treatment and subsequently give rise to new tumors, leading to disease relapse. Often, this means more treatment-resistant and aggressive tumors, resulting in poor patient outcomes in the clinic. Thus, methods to target and eliminate such populations are crucial to improve the efficacy of current therapies in the treatment of breast cancer. One such problematic population is the polyploid giant cancer cells (PGCCs). These morphologically enlarged cells are often multinucleated, with aberrant gene expression. PGCCs have been previously identified in both pre-malignant tissue and patient tumors, with much greater incidence in late-stage disease and after chemotherapy [7,8]. Despite the apparent relationship to more advanced disease, they were often overlooked due to their apparent dormancy and lack of active proliferation. However, recent findings regarding their abnormal cell cycle and chemoresistance have rekindled interest in PGCCs. These studies paint these polyploid giants as a problematic population in terms of both disease recurrence as well as driving agents of cancer progression.

1.2. In-vitro characterization of PGCCs

Although the exact origins of polyploid cells remain shrouded, recent

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findings have postulated that these cells are formed through a mixture of mitotic catastrophe/slippage and cell fusion [9,10]. Regardless of their exact mechanism of formation, it is clear these cells exist as a small but significant and ubiquitous population across virtually all cancer lines as well as solid tumors. More importantly, a variety of external perturbations such as drug treatment and irradiation can dramatically enrich for these cells, selectively enhancing the dominance of this subpopulation through increased formation and selection for these giant cells due to their survival advantage. In terms of their drug resistance, articles from multiple labs [7,11–14] have extensively documented the ability of PGCCs to resist conventional chemotherapeutics, including doxorubicin, vinblastine, and paclitaxel. These cells can survive extreme dosages of these treatments, with over ten-fold the IC-50 values of their parental populations [15]. One proposed theory of their increased resistance is through their unusual amitotic proliferation cycle. PGCCs are capable of proliferation through abnormal cell cycling, dubbed the "giant cell cycle" [12] (Fig. 1A). These giant cells undergo periods of initiation into polyploidy, self-renewal akin to stem cells, termination of polyploidy through budding into daughter cells, and finally stabilization of actively proliferating diploid progenitor cells. The length of this cell cycle is prolonged compared to normal mitosis and may include transient periods of dormancy. We have shown that MDA-MB-231 cells treated with high-concentration (500 nM) of paclitaxel (PTX) for 18 h initially enrich for PGCCs up to 7 days, and then the percentage begins to drop due to budding and generation of daughter cells (Fig. 1B). MDA-MB-231 cells treated with Doxorubicin has been also shown to undergo similar dynamic change in PGCC – initial enrichment of PGCCs followed by rise of daughter cell population [16]. The daughter cells formed from PGCCs have also been shown to be chemoresistant and tumorigenic, capable of giving rise to treatment resistant tumors. This was validated through a series of in-vitro and mouse xenograft experiments [13]. Taken together, PGCCs represent a population of cancer cells that can evade treatment through periods of transient dormancy, and relapse into full blown disease through diploidization into chemoresistant progenitors [17,18].

1.3. *Molecular signatures of polyploidy*

Polyploidy has been observed in both normal mammalian tissue and in more evolutionary primitive phylostrata —prokaryotic, early eukaryotic organisms. Given their aberrant DNA content and chromosomal organization, the molecular profile of PGCCs is extremely complex. This provides added resistance to DNA-damaging agents such as ionizing radiation and platinum drugs, which drive apoptosis through induced DNA damage. Due to the presence of multiple gene copies from chromosomal duplication, PGCCs can resist deleterious damage to certain gene clusters that would normally be cytotoxic. Furthermore, levels of gene expression are also dramatically altered due to their unique chromosomal organization. As a direct result, they present as

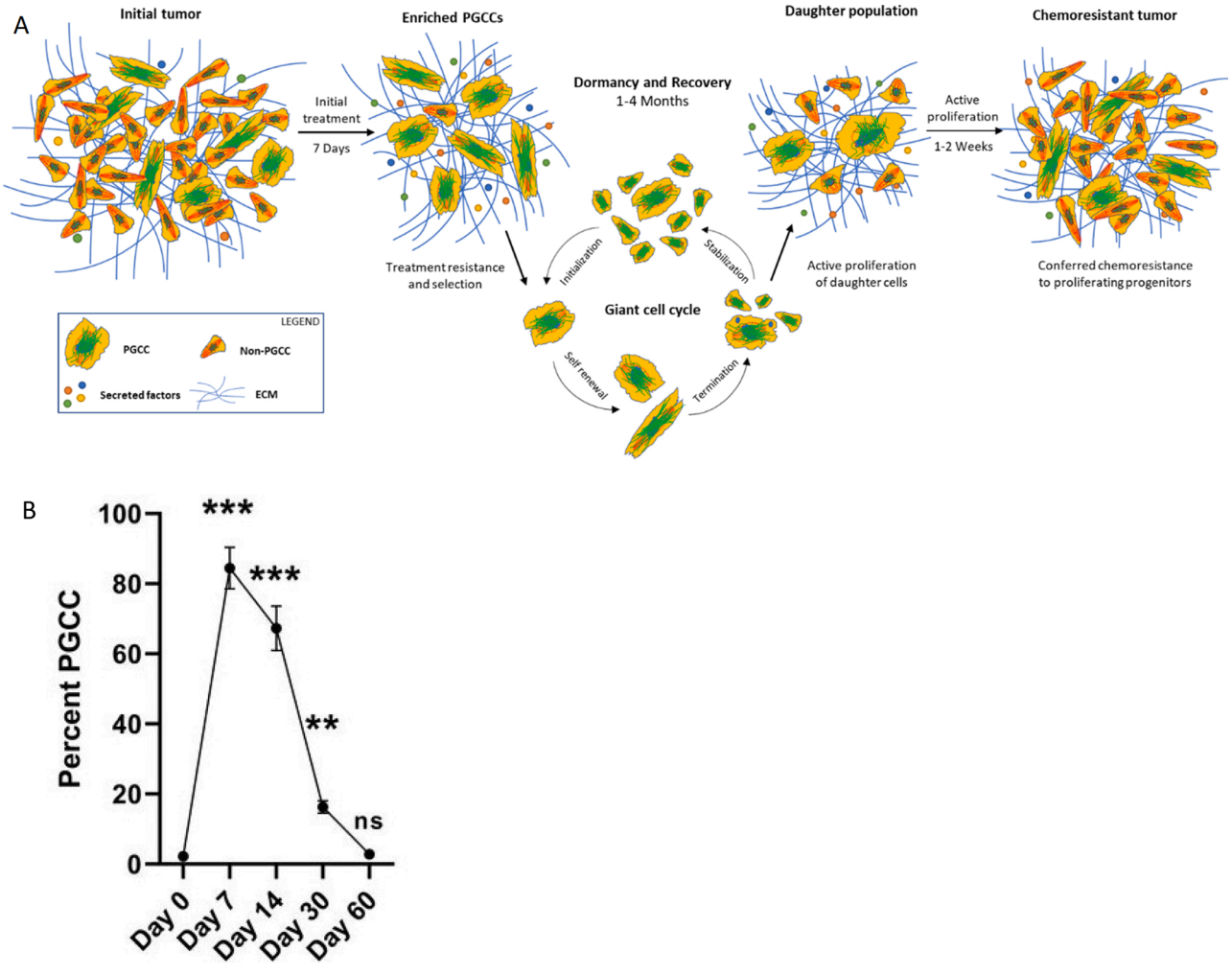

Fig. 1. Role of PGCCs in driving tumor chemoresistance and relapse. (A) Schematic depicting the contributions of PGCCs in disease relapse, from enrichment via preferential survival, entry into dormancy, recovery into actively proliferating daughter cell populations, and finally development into chemoresistant tumor. (B) Time course population analysis of PGCCs after treatment with 500 nM PTX. PGCCs were defined as cells with 2.5 times the nuclear area of control MDA-MB-231, to account for cells undergoing active DNA replication and cell division.

major drivers of intratumoral heterogeneity, convoluting the molecular landscape and act as barriers to effective targeted therapy. This molecular heterogeneity can then be passed down to their progenitors via nuclear budding or bursting of daughter cells, depolyploidization and sub-genome transfer [17]. These processes bypass the mitotic checkpoints that limit DNA damage and often lead to DNA and chromosomal mutations. These mutations can include whole chromosome translocations or regional inversions or deletions. Furthermore, recent studies in phylostratigraphic effect of polyploidy found that epigenetic regulation favors atavistic shift of polyploid cells and phenotypic polyploid cancer cells to the unicellular and early multicellular phenotype [19]. These studies can further elucidate the role PGCC budding and de-polyploidization is typical of more primitive organisms. Taken together, their abnormal means of proliferation can give rise to aneuploidy and genomic instability in their progenitors, which is a hallmark of cancer cells.

Despite the seemingly random nature of their genetic abnormalities, there exists a consistent PGCC molecular signature. It is hypothesized that mutations of caretaker and gatekeeper genes permit survival after mitotic catastrophe and are essential to the formation and survival of these polyploid cells. Furthermore, exposure to drug treatments and irradiation selectively amplify certain loci within the PGCC genome, creating beneficial heterogeneity which can then confer resistance [20]. Numerous studies have shown that PGCCs express cancer stem cell (CSC) associated genes, such as CD44 and CD122, and can differentiate along various lineages [11,14,21,22]. Other studies have reported that radiation induced transcriptional activation of reprogramming genes, including OCT4, SOX2, and NANOG in polyploid breast cancer and lymphoma cells [21,22]. First identified in leukemia patients in 1994, CSCs have been shown through various studies to be highly tumorigenic, multi-drug resistant, and metastatic [23,24]. The CSC-like nature of PGCCs is further corroborated through in-vitro and in-vivo findings, where they found that PGCCs could differentiate along multiple lineages and can resist treatment through temporary phases of dormancy [25]. Subsequently, these PGCCs can also form new tumors in mouse xenograft models, highlighting their tumorigenic potential [11]. Furthermore, molecular profiling of the PTX treatment induced PGCCs at 7days performed by our lab has shown that they express both epithelial and mesenchymal genes associated with mixed EMT phenotype (Fig. 2**A-D**). This mixed or "hybrid" EMT state is characterized by overexpression of both epithelial (such as E-Cadherin) and mesenchymal markers (vimentin, N-Cadherin). Recent literature suggests that contrary to traditional binary views of EMT, cancer cells exist on various points within the E/M spectrum [26]. The ability to transition on this spectrum and exist in a hybrid E/M phenotype allows cancer cells to adapt to their local stresses, increasing their chemoresistance and invasive potential [27–29]. We also found that genes regulating G1/S cell cycle transition, including cyclins E1 and D1 as well as CDK4 were downregulated in PGCCs isolated at 7days after PTX treatment, suggesting that these PGCCs are unable to undergo re-replication and further polyploidization. Other studies using a different model of PGCC isolation (cobalt chloride treatment) have reported increased expression of cyclins and CDK4 [30]. These differences in cell cycle related gene expression further highlight the heterogeneity and dynamics of PGCCs. Similar to the E/M spectrum, PGCCs can likely exist in multiple states in cell cycle: it can oscillate from senescence-like state of cell cycle arrest to reprogrammed state of active replication. Furthermore, they often have deregulated expression or mutations of tumor suppressor proteins such as p53, APC, and oncogenes such as Myc and Ras, which allow these giant cells to survive mitotic failure events and resist apoptosis [30–36]. In addition, they have been shown to have over expression of CSC as well as a hybrid EMT signature, contributing to their chemoresistance and

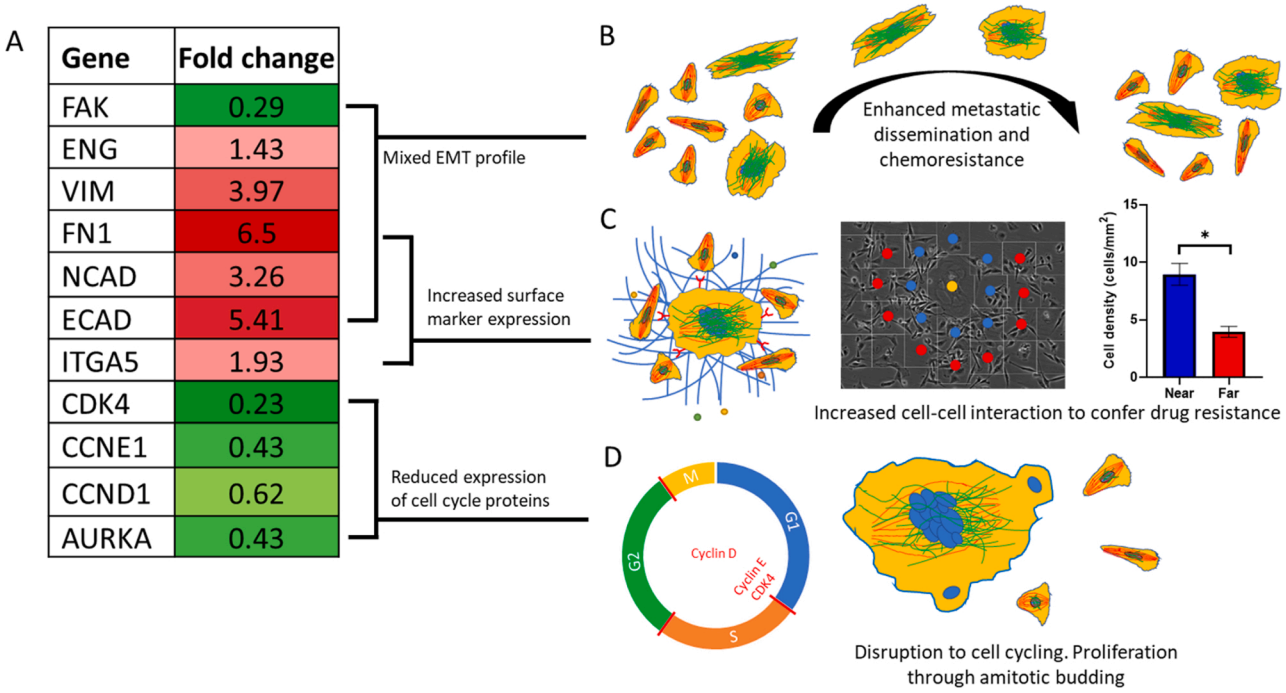

Fig. 2. Molecular characterization of polyploidal cells. MDA-MB-231 cells were treated with 500 nM PTX for 18 h to induce polyploidy, and subsequently allowed to recover for 7 days. Resulting populations of high-purity PGCCs were collected for qRT-PCR analysis and comparison with untreated MDA-MB-231 control cells. (A) Quantitative RT-PCR analysis of PGCC gene expression demonstrated a mixed-EMT profile, which consists upregulation of both epithelial markers as mesenchymal markers. Furthermore, PGCCs had increased expression of surface markers and reduction in cell cycling proteins. (B) A hybrid EMT profile is associated with increased metastatic dissemination and chemoresistance. (C) Increased cell-cell interaction due to surface marker overexpression in PGCCs, leading to increased cell density near PGCC surface. Bright field images of MDA-MB-231 cells were taken and manually analyzed for PGCCs (dimension: 891um x 653um). Images centered on PGCCs were sub-divided into equally spaced sections and assigned as either "near" (within 1 section) or "far" (greater than 1 section) relative to PGCC position. Total cells within each section were counted and quantified. (D) Reduction of cell cycle proteins leads to slow or halted mitotic cell cycling, instead driving proliferation through amitotic budding.

tumorigenic potential. In sum, polyploid cells have a highly complex and heterogenous molecular profile, comprised of a hybrid of multiple phenotypes, making it difficult to target these cells that often evade treatment.

2. The distinct PGCC biophysical phenotype

Mechanical forces acting on the tumor are potent regulators of invasive cancer cell behavior [37–39]. Tumors are under compressive stress from rapidly proliferating cancer cells, recruited stromal cells, and extracellular matrix components [40–43]. Fragile tumor blood vessels and lymphatics are often compressed and leaky in this crowded environment. This leads to high interstitial fluid pressures that further contribute to the compressive solid stress in the tumor [44–47]. The surrounding tissue exerts reciprocal force at tumor periphery to balance forces from the growing tumor. However, as the cancer progresses and these forces continue to accumulate, cancer cells undergo adaptations that allow them to invade the surrounding environment [44,48]. These adaptations often involve alterations in cytoskeletal organization that serve to maintain the viability of cells in these high-pressure environments and promote invasion into the surrounding matrix. In addition to maintaining cell viability against exerted forces, the cytoskeleton is involved in the highly conserved pathway of mechanical crosstalk, which is crucial for the transmission of signals to and from the surrounding extracellular matrix (ECM) [49]. This process allows for the modulation of gene expression and the alteration of cell behavior depending on changes to the local microenvironment [50–52]. Thus, cancer cells can actively sense, adapt and alter their microenvironment. These alterations can include raised stiffness due to increased ECM deposition and crosslinking and malignant transformation of subpopulations of cancer cells, promoting increased invasion and metastatic potential [53,54]. In both of these aforementioned processes, the cytoskeletal network of the cell is a crucial mediator. For example, the activation of actin-associated Rho/ROCK signaling directly influences actomyosin contractility, which often results in malignant transformation and stiffening of both the cell as well as the surrounding tissue [55–57]. In this tense force situation, vimentin intermediate filaments have been shown to serve a cytoprotective role due to their unique strain-hardening properties, protecting the cell nucleus and cytoplasm against repeated and large stresses [58–60]. Furthermore, they have also been shown to polarize cell migration due to their relatively stable nature and long macrostructure half-life. By associating with actin and microtubule networks through linker proteins, vimentin can polarize the entire cytoplasmic organization of the cell, increasing directionality of cell movement and polarizing its migration [61,62]. Thus, it is extremely important to closely study the organization of individual cytoskeletal networks in order to understand the underlying drivers of cancer cell behavior. This is especially true in PGCCs, where their increased size and migratory persistence demand irregular organization and altered expression of these filamentous proteins.

2.1. Unique cytoskeletal organization of PGCCs

One of the most distinct features of PGCCs is their massively enlarged size. Volume reconstruction analysis of these giant cells revealed on average over a 15-fold increase in cytoplasmic volume compared to non-PGCCs [63]. Their enlarged size was supported by significant differences in the expression and organization of cytoskeletal components. During our molecular characterization of PGCCs, we identified increased actin fibers through immunofluorescent staining and significant upregulation in the expression of RhoA, which is important in actin myosin contractility [15]. Previous studies have shown that Rho/ROCK regulates actin network organization as well as cytoskeletal tension, driving tissue stiffening and malignant transformation [55]. Furthermore, the actin bundling of α-actinin and crosslinking through filamins can give rise to actin stress fibers. These stress fibers connect the cytoskeleton to the ECM via focal adhesions and can directly affect cytoplasmic stiffness. Taken together, disruptions and alterations to the Rho-ROCK signaling pathway and actin cytoskeletal organization could contribute to malignant transformation of cancer cells and their local surroundings [64–66]. To confirm the observed differences in actin organization, we quantified phalloidin-stained actin stress fibers in PGCCs vs non-PGCCs in the parent population [15]. Indeed, MDA-MB-231 polyploid cells had higher levels of actin bundling, with longer and thicker stress fibers (Fig. 3**A-B**).

Our molecular signature also showed vimentin is upregulated in PGCCs compared to non-PGCCs. Vimentin plays a critical role in cell migration and invasive cancers, so it is crucial to examine in the context of cancer metastasis. Traditionally, vimentin has been viewed as a fundamental regulator of cell architecture, nuclear arrangement, gene expression and chromatin condensation. The coiled-coil alpha-helical structure of vimentin dimers lends to vimentin's unique biophysical properties. Studies of vimentin filaments show that they are pliable until tensile strains exceeding 100 % of their original length, at which point vimentin undergoes strain hardening. This unique feature allows vimentin to play a cytoprotective role, permitting small and frequent morphological changes but protecting against large deformations and repeated stresses. Vimentin filaments have also been shown to support the overall structure of the cell, due to their relatively stable macrostructures [67]. This is extremely relevant in the context of PGCCs, as they would require significant structural support to maintain their larger volume. Furthermore, PGCCs migrating in confluent monolayers or 3-D environments would undergo significant deformations as they translocate through tight spaces. These large deformations would require heavy reliance on their vimentin network to protect cytoplasmic structures, organelles and nuclear structure. When we performed immunofluorescent staining of PGCC vimentin networks, we saw increased expression of vimentin as well as more distributed vimentin intermediate filament networks. Compared to the bright perinuclear puncta observed in non-PGCCs, polyploid cells had extensive vimentin intermediate filament networks spanning the entire cell, likely needed as support structures for cellular integrity. Indeed, when we collapsed their vimentin structures using low dose acrylamide or complete knockdown using siRNA, we saw a greater reduction in PGCC cell volume in comparison to non-PGCCs. This suggests PGCCs depend more heavily on vimentin for cytoskeletal structural support compared to non-PGCCs. Lastly, the expression and organization of microtubules also plays a large role in cancer progression. These highly dynamic structures have been associated with mitosis, cellular trafficking and migration [68]. The unique phenotype and morphology of PGCCs likely demands altered organization of its microtubule networks. We previously found distinct alterations in the microtubule networks of PGCCs, characterized by the lack of apparent microtubule polarization and the presence of multiple microtubule organizing centers (MTOC) [15]. Previous studies have shown that the presence of multiple MTOCs in numerous cancers with chromosomal instability, which drive mutations and sustained DNA damage [69,70]. Taken together, these differences in key cytoskeletal structures of actin, vimentin and microtubules result in distinct alterations to the PGCC biophysical phenotype. The increased actin stress fiber formation and vimentin intermediate filament polarization observed in PGCCs drive key behavior differences that can play important roles in PGCC invasion and metastasis.

2.2. Biomechanical measurements of polyploidy

Cytoskeletal organization is central to cell biophysics, including nuclear and cytoplasmic stiffness, traction force generation and cell migration [71,72]. Actin cytoskeletal proteins are distinctly altered in their organization and expression, suggesting PGCCs may also have altered biophysical properties. Using intracellular multiple particle tracking (MPT), we recently characterized cytoplasmic and nuclear rheology of PGCCs. This technique involves tracking the Brownian

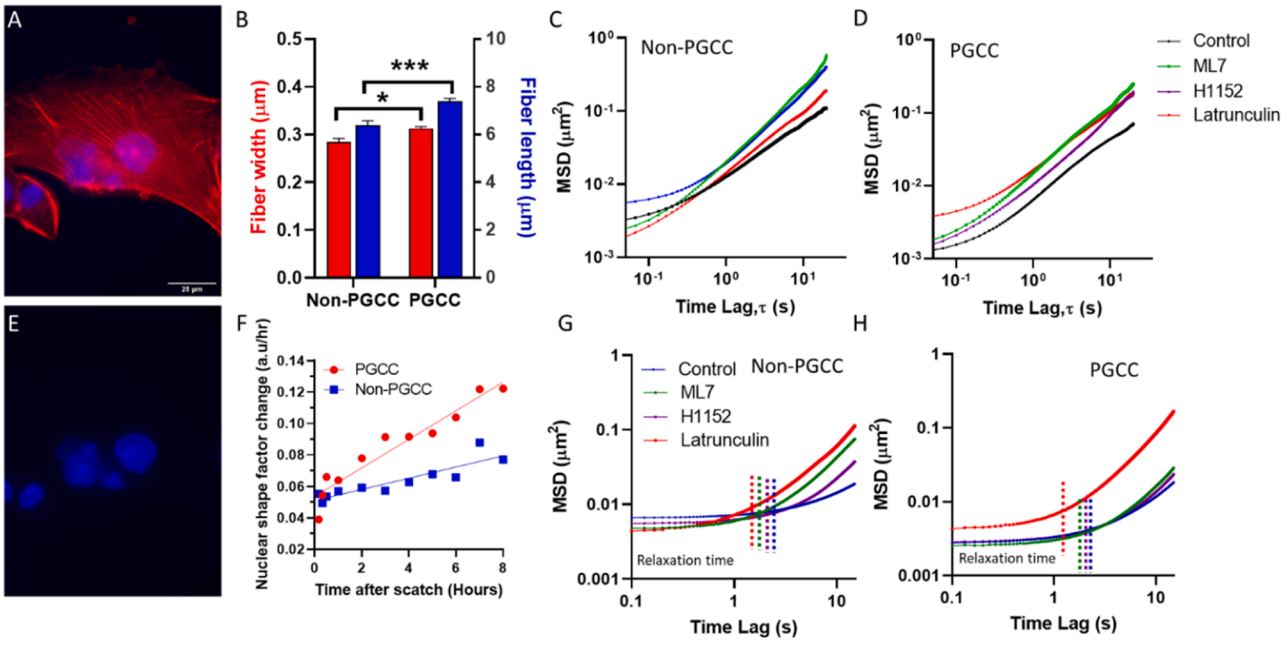

Fig. 3. Biophysical analysis of PGCCs. (A) Rhodamine-phalloidin staining of PGCC actin network, highlighting the increased actin stress bundles and organization of overall actin structure. (B) The length and width of actin stress fibers were measured via Cell profiler using a custom pipeline. Quantification of actin fibers shows both increased stress fiber width and length in PGCCs compared to non-PGCCs. 200-nm fluorescent carboxylate particles were ballistically injected into cell cytoplasm for nanoparticle tracking analysis. Derived particle motions provide insight on the biophysical properties of the cell cytoplasm. The ensemble averaged MSDs of particle motion in non-PGCCs (C) and PGCCs (D) show inherently more constrained particle movement in PGCCs compared to non-PGCCs. Since cytoskeletal mechanics are mainly governed by the actin cytoskeletal network, this result was attributed to the increased actin stress fiber density. To analyze contributions of the actin network on PGCC biophysics, polyploidal and non-polyploidal MDA-MB-231 cells were treated with inhibitors targeting actin network assembly as well as the Rho-Rock signaling pathway, which governs actin contractility. Namely, MDA-MB-231 PGCC and non-PGCCs were treated with H1152, Latrunculin, and ML7 and analyzed for differences in their biophysical phenotype. Targeted inhibition of the Rho-Rock signaling pathway and actin disruption increased particle motion preferentially in PGCCs, indicating increased contribution of the actin network to PGCC cytoplasmic stiffness compared to non-PGCCs. Similar to cytoplasmic nanoparticle tracking analysis, live tracking of heterochromatic foci of Hoechst stained PGCC and non-PGCC nuclei (E) show increased deformability during scratch wound migration (F). (G-H) MSD analysis of heterochromatin bright spots reveal inherent stiffness in PGCC nuclei. Again, we treated with inhibitors targeting the actin network to show the reduced nuclear particle motion in PGCCs was regulated by a dense network of actin filaments, which formed a perinuclear actin cap in PGCCs.

motion of fluorescently labeled nanoparticles embedded in the cell cytoplasm or nucleus. The time dependent particle mean squared displacements are calculated from the particle locations and the ensemble-averaged mean squared displacement (MSD) (which represents the time-dependent average MSD for all of the particles) is used to determine micromechanical properties (shear modulus, viscous and elastic properties, and relaxation time). Because the motion of the embedded particles is constrained by local cytoskeletal elements, the actin filamentous network plays a critical role in regulating cytoplasmic stiffness. Indeed, when we analyzed the MSDs of particles embedded in PGCCs, we found lower individual particle MSDs, indicating the particle motion was more constrained in the PGCC actin cytoskeleton (Fig. 3**C-D**). This restricted particle motion corresponds with increased stiffness, which would support the enlarged morphology of giant cells and help them to resist mechanical forces in the tumor microenvironment. Furthermore, particle motion was more heterogeneous in PGCCs (in comparison to non-PGCCs) as indicated by increased coefficients of variation at multiple time points. This was expressed as both intracellular regions of soft and hard rheology, as well as large differences between cells, all contributing to significant tumor heterogeneity. To confirm the role of actin in their inherent cytoplasmic stiffness, a variety of small molecule inhibitors targeting the actin cytoskeletal network were deployed. Treatment with Latrunculin-A (actin depolymerizing agent), ML7 (myosin light chain kinase inhibitor), and H1152 (RhoA--kinase inhibitor) significantly increased the particle motion (or MSD) in PGCCs much more than non-PGCCs (Fig. 3**C-D**). This suggests that altering the actin network reduces stiffness in the PGCC cell cytoplasm. ML7 and H1152 were used to block actin myosin contractility, which is a critical mechanism of actin-based motility. This suggests that polyploid cells rely heavily on their increased actin networks to maintain stiffer rheological properties. Similar to cytoplasmic MPT, chromatin tracking involves tracking the motion of brightly stained heterochromatic foci in Hoechst -stained nuclei to obtain nuclear biomechanical properties. PGCCs had on average lower particle motion within their nucleus, indicating an overall stiffer structure. This is most likely due to the fact that these cells are not actively dividing and likely remain transcriptionally inactive, so their chromatin remains in the more condensed heterochromatin state. Alternatively, their increased stiffness could also be due to steric constraint since cells have increased copies of chromosomal DNA packed into their enlarged nuclei. Like their cytoplasmic properties, PGCCs had much higher levels of innate heterogeneity, owing to their multinucleated and abnormal nuclear structure. In addition to particle MSD, another important parameter in the analysis of nuclear mechanics is the nuclear relaxation time. This relaxation time represents the nuclear transition from elastic (resistant to deformation) to viscous (deformable) behavior, as shorter relaxation time signifies more deformable material. Surprisingly, PGCC nuclei demonstrated a shorter relaxation time, suggesting a faster transition to a viscous behavior and more deformability. These findings highlight key differences in the dynamics of PGCC nuclear mechanics, with a stiffer yet more deformable nuclei (Fig. 3**E-H**). This seemingly conflicting nature arises from a multitude of factors, including their multinucleated phenotype as well as the ability to alter the shape of their nuclei via direct interactions with their unique actin network. These multi-nucleated polyploid cells are able to alter their nuclear macrostructure by rearrangement of individual “lobes” of nuclei, resulting in

large shifts in overall nuclear shape and distribution. Furthermore, PGCCs are also able to compress and reshape their nuclei, through a combination of actin and intermediate filament networks coupled to the nuclear envelope [73–75]. This is especially interesting in the context of cancer aggression, where the rearrangement of nuclear shape is essential for invasion [76,77]. In 3-D environments and confluent monolayers, the nucleus acts as the barrier to cell movement, being by far the stiffest component of the cell [78]. This means that in order for PGCCs to migrate, they must be able to arrange and deform their multinucleated structures. Indeed, when tracked over the course of 9 h during migration in confluent monolayers, PGCCs were more readily able to rearrange their multinucleated structures and deform the nucleus. When squeezing past neighbors, they can arrange their multinucleated nuclear structure along a single axis, forming a narrow string of subnuclear structures. This reduces the width occupied by their nuclear material, allowing them to squeeze through with ease [15,63].

2.3. The persistent migratory phenotype

Actin filaments assemble into contractile stress fibers, which can drive cellular stiffening, malignant transformation and exert traction forces onto the surrounding ECM. Subsequently these traction forces propel the cell forward in the direction of movement. The amount of traction force generation and resulting migration is directly related to the size and numbers of the contractile stress fibers [37,79]. Because PGCCs have thicker and longer stress bundles, they are likely to exert higher contractile forces, leading to increased migration. To analyze exerted forces, traction force microscopy can be utilized to measure the amount of contractile pressure that is being applied to the surrounding environment by the cell. Cells are seeded on a fluorescent nanoparticle embedded polyacrylamide gel of a known stiffness and allowed to attach. Initial particle positions are recorded via high-resolution imaging followed by subsequent trypsinization and detachment of the cells. Another image is taken after detachment and the displacement of the particles is used in conjunction with the known stiffness to determine exerted traction forces. Through this method, our lab determined that PGCCs had on average 2 to 5-fold higher maximum exerted traction forces, compared to non-PGCC counterparts (Fig. 4).

In addition to the transmission of force to the ECM, another important component in cancer cell migration is the polarity or directionality of movement. A cell which has a persistent path and directionality can traverse a longer net distance a cell moving randomly. Thus, the polarization of cytoskeletal features and migratory persistence is crucial to the invasion of individual tumor cells into the surrounding matrix and subsequent metastasis. A key driver of cell polarization is the intermediate filament vimentin, important in front-to-back polarization of the cell [61,62]. Due to their relatively stable macrostructure, vimentin filaments also act as guide tracts for the arrangement of cytoskeletal elements with shorter half-lives. Previous research has shown that vimentin intermediate filaments directly couple with microtubule networks and can even provide a template for guiding microtubule growth at the plus end. Furthermore, by constraining the spaces where actin fibers elongate, they can also facilitate directionality in actin treadmilling to polarize cells during migration. Taken together, the unique features of vimentin not only serve as protective structure for the nucleus but are crucial in maintaining persistence during cell migration. When we tracked the movement of PGCCs in random migration and scratch wound assays, we found that these polyploid cells have much higher rates of migratory persistence compared to non-PGCCs. During the course of 12 h in a scratch wound, more PGCCs would squeeze past their neighbors and migrate into the middle of the scratch, resulting in a higher proportion of PGCCs within the scratch at the end of 12 h (Fig. 4**A-C**). Furthermore, polarity analysis of their vimentin intermediate filament revealed an increase in the fraction of vimentin that was polarized in respect to the nucleus in the direction of movement. This meant that their vimentin intermediate filament network plays a predominant role in dictating the migratory direction over longer periods. To confirm that the increased migratory phenotype of these polyploid cells and their persistence in motility was due to their upregulated vimentin network, our lab performed acrylamide treatment and siRNA knockdown of vimentin expression. Disruption of PGCC vimentin organization and expression significantly decreased their migratory phenotype compared to non-PGCCs, confirming their reliance on the uniquely distributed vimentin intermediate filaments to polarize their

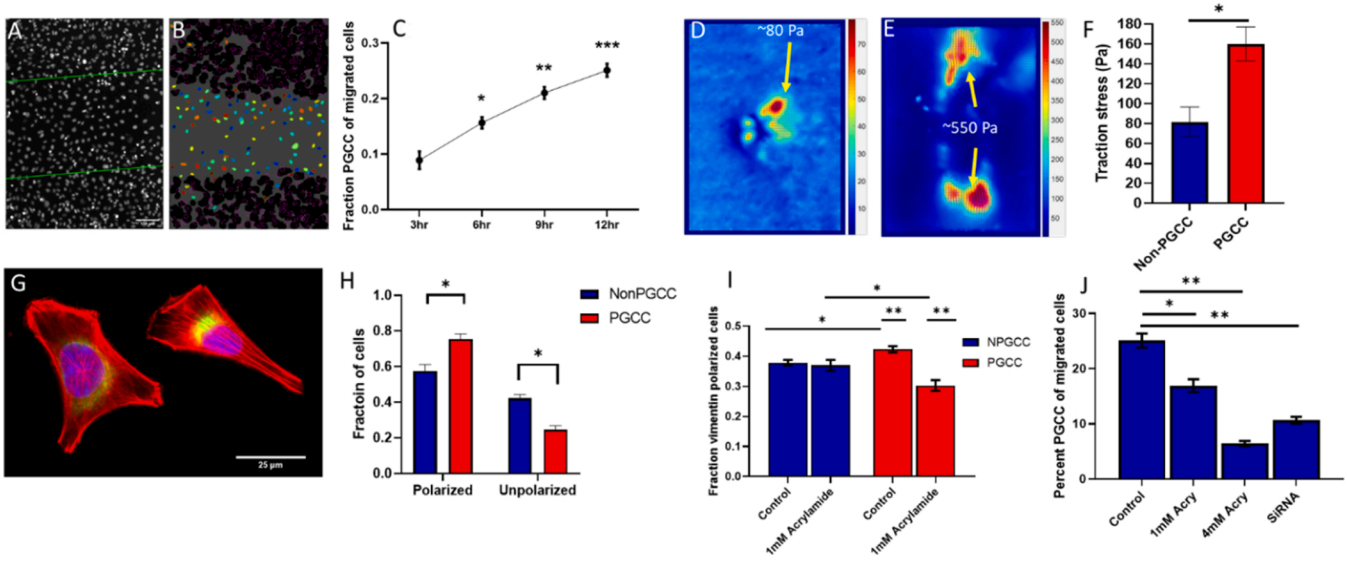

Fig. 4. The migratory phenotype of PGCCs. (A) Hoechst stained nuclei of MDA-MB-231 cells during scratch wound at 0 h (green lines) and 12 h after scratch. (B) Cell profiler segmentation and analysis of migrated nuclei for high throughput automated analysis. (C) Quantification of the percentage of PGCCs in the migrated cell population shows preferential migration of PGCCs relative to non-PGCCs into the scratch wound over time. Traction force microscopy analysis of non-PGCCs (D) and PGCCs (E) highlights greatly increased maximum exerted traction forces by PGCCs (F) on 10 kPa polyacrylamide substrates. (G) Analysis of vimentin polarization revealed greater fraction of polarized vimentin in PGCCS compared to non-PGCCs (H). (I) Treatment with low dosage acrylamide significantly reduced the polarization of vimentin preferentially in PGCCs. (J) Disruption of vimentin polarization via acrylamide or siRNA knockdown significantly decreased the PGCC migratory phenotype, suggesting vimentin polarization plays a large role in their invasive phenotype.

migration (Fig. 4**G-J**). Overall, PGCCs represent a highly migratory and aggressive population of cancer cells, capable of invading the local stroma through their vimentin-driven invasive phenotype. This is highly problematic in the context of patient outcomes, as distal metastasis is the biggest driver of cancer-related mortalities [80,81].

3. PGCCs in the greater tumor microenvironment

Previous studies on PGCCs primarily focused on chemoresistance and tumorgenicity; however, it has been well established that the interaction of cancer cells with the surrounding tissue and their neighbors could play a large role in cancer progression and metastasis. Cancer-stromal cell interactions have been previously characterized as key drivers of drug resistance and metastasis, either through secreted factors or direct cell-cell interactions [8,82,83]. Previous studies have shown that mesenchymal stem cells (MSCs) can increase cancer cell proliferation and promote metastasis through induced EMT via secreted factors such as TGF-β, IL6 and IL [83–85]. Furthermore, direct cell-cell interaction between MSCs and cancer cells can confer drug resistance through integrin signaling [86,87]. Indeed, the expression of integrin alpha 5 is commonly altered in cancer cells, and the activation of integrin signaling has been linked to increased survival and malignant transformation. Across numerous experiments, polyploid cells have been seen in close proximity to non-PGCCs, in both naïve and PTX treated conditions (Fig. 3C). Given this observation and their molecular overexpression of surface markers, it is likely that these polyploid cells have increased cell-cell interactions with surrounding cancer cells, akin to an MSC [88]. Additionally, therapy induced polyploidal cells in the tumor microenvironment display transient senescent cell like behavior including increased b-gal expression, and secretory phenotype as well as previously described reprogrammed pluripotent cell like behavior [89,90]. Both of these attributes in PGCCs likely alter their interaction in the microenvironment. To fully determine the contributions of PGCCs in driving tumor drug resistance and metastasis, these direct interactions must be investigated.

3.1. Stromal-like interactions of PGCCs

When quantified, this clustering phenomenon results in over 2-fold the cell density near the polyploid cells compared to far away. This suggests that PGCCs have unique expression of surface markers that directly interact with other cancer cells, akin to a stromal-like behavior. Through qRT-PCR analysis of surface markers, PTX-induced PGCCs have been found to have higher expression of E-, N- and OB-cadherins, as well as fibronectin and integrin alpha 5. It is likely that these polyploid cells play a stromal cell role in the context of conferring chemoresistance, through direct interactions with non-PGCCs. Furthermore, expression of chemo-attractants and secreted chemokines such as IL-8 and IL-6 would likely lead to chemotaxis of surrounding cells and immune cell recruitment. Indeed, it is likely that these PGCCs can give rise to a chemoresistant phenotype in nearby cells and their budded daughter cells, by harboring these neighboring cells as they develop drug resistance. This is especially critical in the context of cancer treatment and relapse prevention, as having pockets of chemoresistant cells with highly tumorigenic potential will certainly lead to cancer relapse and patient mortality. Thus, it is imperative to limit paracrine and direct cell-cell interactions in order to mitigate the effects of PGCCs on other cells in the tumor microenvironment. In fact, numerous adjuvant cancer drugs often target stromal cells due to their harmful effects on the tumor microenvironment [91]. These therapeutics could potentially be effective against PGCCs, preventing their contributions to malignant cancer transformation.

4. Concluding remarks

Previous research has shown that cancer relapse is often the result of a drug-resistant subpopulation which gives rise to new tumors after the initial treatment. PGCCs are one such subpopulation, which has previously been observed in vivo and is especially prominent in high-grade, late-stage and treatment resistant cancers. Recent studies have demonstrated the relevance and importance of these PGCCs in cancer progression [11,12,14], alluding to the critical role these PGCCs play as the reservoirs of chemoresistance [13,15,63]. They not only can resist treatment but can also confer a drug resistant phenotype to surrounding cells through direct cell-cell interactions akin to stromal cells. Through biomechanical measurements and various functional assays, PGCCs have also been found to exhibit a unique biophysical phenotype. This consists of a stiffer cytoplasm and nucleus, primarily driven by the upregulated actin cytoskeletal network. Although their nucleus is stiffer, they are more deformable. This feature in conjunction with the increased ability to generate traction forces means PGCCs can invade surrounding stroma with ease, posing as a highly invasive subpopulation. Furthermore, the differential and polarized organization of vimentin further attenuates their migratory phenotype, promoting increased invasion and metastasis. This also implies that compared to non-PGCCs, PGCCs rely heavily on their vimentin intermediate filament network to maintain polarity in migration. Under normal circumstances, migratory persistence is driven by the polarized MTOC position in regard to the direction of cell migration [92,93]. However, with the high levels of nuclear disorganization and mitotic irregularities in PGCCs, the microtubule polarizing effect is disrupted [15]. It is likely that vimentin is then recruited into this role to compensate for the loss of MTOC polarity. In addition to its part in facilitating polarization, vimentin also has an enhanced cytoprotective role. PGCCs utilize vimentin to resist axial strain and protect their nucleus during periods of large deformation during migration.

In sum, recent studies have elucidated the unique molecular and biophysical signature of PGCCs and underscored the importance of this previously overlooked subpopulation. These cells are linked to cancer progression, treatment resistance and relapse. These PGCCs exhibit increased intracellular heterogeneity, a characteristic correlated with high levels of metastasis and aggression. With the increased prominence of PGCCs in more aggressive tumors or after treatment with chemotherapeutics, their role in cancer progression is extremely important in the context of treatment and disease progression. Future directions should focus on finding targeted therapies towards key differences in their molecular and biophysical profile. For example, small molecule inhibitors could target the RhoA-ROCK1 pathway as an adjuvant therapy, mitigating the biomechanical phenotype that supports their increased size. Alternatively, the targeting of vimentin crosslinking could prevent their polarization effect, diminishing the migratory abilities of PGCCs and reducing their invasive potential. Lastly, work should be done in terms of forming a panel of biomarkers unique to PGCCs according to their molecular profile. This will enable the identification of PGCCs in patient samples and can potentially be used as a prognostic marker for clinical diagnosis since increased numbers of PGCCs have been seen in more aggressive cancers.

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Declaration of Competing Interest

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