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Polyploid giant cancer cells: unrecognized actuators of tumorigenesis, metastasis, and resistance

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Abstract

Cancer led to the deaths of more than 9 million people worldwide in 2018,[1] and most of these deaths are due to metastatic tumor burden. While in most cases we still do not know why cancer is lethal, we know that a total tumor burden of one kilogram – equivalent to one trillion cells – is not compatible with life. While localized disease is curable through surgical removal or radiation, once cancer has spread, it is largely incurable. The inability to cure metastatic cancer lies, at least in part, to the fact that cancer is resistant to all known compounds and anti-cancer drugs. The source of this resistance remains undefined.[2] In fact, the vast majority of metastatic cancers are resistant to all currently available anti-cancer therapies, including chemotherapy, hormone therapy, immunotherapy, and systemic radiation. Thus, despite decades – even centuries – of research, metastatic cancer remains lethal and incurable.[3] We present historical and contemporary evidence that the key actuators of this process – of tumorigenesis, metastasis, and therapy resistance – are polyploid giant cancer cells.

Keywords

Polyploid giant cancer cells; keystone species; therapeutic resistance; stemness; metastasis; cancer ecology

The term *cancer* is derived from the Greek word for crab, used by Hippocrates to describe solid malignant tumors, circa 400 BC. The word *metastasis*, from the Greek for "displacement," was formally described by French physician Joseph Récamier in 1829 in his treatise Recherches sur le traitement du cancer (translated *Research on Treatment of Cancer*).[4] By this time, the idea that cancer spreads from its primary site was well appreciated and cell theory was established and accepted, though the routes or origins of

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metastases were not clear. In 1889, Stephen Paget, a surgeon at the West London Hospital and the Metropolitan Hospital in London, UK, performed an autopsy series of 735 women who died of fatal breast cancer.[5] In this *Lancet* publication, he addressed the common theories of metastasis held by his colleagues and presented his data that demonstrated a clear pattern of metastatic spread. His now-famous "seed and soil hypothesis" remains the framework for all modern cancer metastasis research: "… every single cancer cell must be regarded as an organism, alive and capable of development. When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil." In concluding this landmark paper, Paget states, "The best work in the pathology of cancer is now being done by those who…are studying the nature of the seed."[5]

In modern cancer biology, it is well accepted that metastatic spread is a stochastically rare event; the overwhelming majority of cells that leave the primary tumor will not establish a secondary tumor.[6] Likewise, we also know that patients with metastatic disease, even with an initial response to systemic therapy, will eventually fail and their disease will recur. Tumor burden falling below levels of radiographic and biochemical detection indicates that the resistant tumor is derived from a single or a few cancer cells that are intrinsically resistant or develop resistance in response to therapeutic selective pressure.[3] This phenomenon of tumor growth from a single or a few cells is supported by phylogenetic analysis.[7–9] Importantly, it appears that both metastasis and therapeutic resistance is mediated by only one or a few cells.

To better understand cancer, tumors may be described and modeled as ecosystems, with cancer cell species co-existing in a complex habitat with host cell species.[6,9–18] The cancer cells, the body's normal cells, and the tumor microenvironment in which they reside and influence make up the cancer ecosystem. In many ecosystems, the community structure and ecosystem integrity are dependent on a single and often low-abundant species termed the keystone species.[13,19] Keystone species are named after the architectural keystone of an arch. If the keystone is removed, the arch – or the ecosystem – collapses. Keystone species exert a disproportionally large effect on the ecosystem relative to their abundance.[19] While there are relatively few individuals within the keystone species in any given community, they occupy a unique and nonredundant niche within the ecosystem – they are not replaceable. Examples of keystone species include the wolves of Yellowstone and the elephants of the Serengeti. Loss of these keystone species had a cascading negative effect on all the other species of the ecosystem, causing fundamental changes and even collapse of the ecosystem structure.

We propose here that the cancer ecosystem is dependent upon a keystone species: a rare population of cells that has the capacity to survive the harsh conditions of the of the tumor microenvironment (e.g., hypoxia, low nutrients, low pH), to metastasize, and to mediate therapeutic resistance by surviving treatment and then repopulating tumors with resistant cancer cells. While these keystone cancer cells survive, metastatic cancer will remain incurable. If we can identify and eliminate the keystone cancer cells, the tumor ecosystem will collapse, making the bulk tumor cells vulnerable to traditional therapies, opening the door for the opportunity for cancer cure. These rare keystone cancer cells, however, remain undefined.

For centuries, physicians and scientists could only observe the natural history of cancer within a patient, without understanding the basic units of the disease: cancer cells. With the invention and wide adoption of the microscope, cancer biologists were finally able to see what cancer cells looked like – to truly study the nature of the seed. With the discovery of cells in 1665 by Robert Hooke, the field of cell biology was born. Johannes Müller, a pioneer in tissue microscopy and histology, published some of the first descriptions and illustrations of cancer cells in his 1838 book *Ueber den feinern Bau und die Formen der krankhaften Geschwülste* (translated: *On the Finer Structure and Form of Morbid Tumors*) (Figure 1A).[20] By 1839 cell theory was formally codified, attributed to plant biologist Matthais Schleiden and Müller's students Theodor Schwann and Rudolph Virchow, and has served as the basis of all modern cell and molecular biology.[21] Virchow, the "father of pathology" and cancer and metastasis biologist, recorded further descriptions of cancer cells in his book Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebenlehre (translated: *Cellular pathology as based upon Physiological and Pathological Histology*), based on his lectures given to the Pathological Institute of Berlin in 1858 (Figure 1B).[22,23]

These discoveries made possible through advances in technology gave the cancer research community the ability to observe and record the phenotypes of cancer cells within primary tumors and metastatic lesions, but early studies were limited to autopsy studies and static tissue sections. The advent of cell culture enabled researchers to observe, perturbate, and phenotype cancer cells over time. George Gey and Margaret Gey successfully isolated cervical cancer cells from Henrietta Lacks in 1951 to generate the first cell line, HeLa, still in use today.[24] Since that time, hundreds of cancer cell lines have been isolated or generated through genetic transformation, enabling new discoveries in cancer cell and molecular biology, as well as advancements in anti-cancer therapies.

Despite all of these discoveries - from the description of the progression of the disease and necessity of the "seed and soil" in 1889,[5] to the visualization and description of cancer cells from patient tumors in 1838–1858, [20,22] to the widespread use of cancer cell lines *in vitro* starting in 1951[24] – we still do not understand, nor can we cure, metastatic cancer. What have we missed?

Prostate cancer biologist Dr. John Isaacs often reminds us that "The most powerful tool we have is our eyes." Have we stopped seeing what is under the microscope? We are blinded by the assumptions of what we expect to see when we look at cancer cells: monolayers or spheroids of more-or-less differentiated epithelial-like cells. Looking through the microscope, at histological sections or cell cultures, we pick out nuclei and cell borders, search for regular patterns of cell size and morphology. We have been trained for generations to dismiss aberrant cells as artifact of the technique or protocol. ResearchGate, the social media forum that allows scientists to seek advice from other researchers worldwide, is peppered with questions about unusual cells in culture. Posters typically respond that the cells are artifact of some external pressure (e.g., old media, over confluence, loss of CO_2 conditions, viral manipulation) or are irreversibly senescent cells that will not survive passaging. Even in our cartoons describing cancer progression and the metastatic cascade, the cells follow a uniform prototype – cuboid for proliferative cells, spindle-shaped for

invasive cells. This is what we teach and this is what we learn. While the stochastic data we have indicates that the critical mediators of lethal and incurable disease appear to be a rare population, we only base our observations on the majority population.

In looking at the hand-drawn illustrations of Müller and Virchow, it is immediately apparent that they observed the inherent cellular heterogeneity of a tumor. While Müller's hand-drawn illustrations are dominated by the typical more-or-less differentiated epithelial-type cells, there are examples of giant multinucleated or large nucleated cells that he specifically highlights (Figure 1A).[20] Virchow described the heterogeneity of cancer cells in his volume as "… curious bodies, provided with large nuclei and nucleoli, which are described as the specific, polymorphous cells of cancer." His illustration of the cancer cells shows great phenotypic heterogeneity, including very large cells with multiple nuclei (Figure 1B).[22,23]

These cells, few in number but persistent within cancer cell populations, may be a cancer keystone species. Close examination of any cell culture flask of any solid tumor type will reveal similar non-typical cells that are morphologically distinct (i.e., non-cuboid and non-spindle shaped) cells with large cytoplasmic region and high DNA content as a single large nucleus or within multiple nuclei. Indeed, a polyploid giant cancer cell is evident in the first published photographs of HeLa cells, the first cancer cell line developed (Plate 39),[25] and this rare population of cells persists today (Figure 1C).

Polyploid giant cancer cells (PGCCs) were observed and recorded at least 180 years ago, and have been visualized in cell culture, the workhorse of cancer cell biologists, for 65 years. The formation of PGCCs following therapeutic intervention, including chemotherapy and radiation, and upon conditioning in hypoxia, simulating the tumor microenvironment, has been described in the literature. Most measures of cell response to therapy, including dose response curves generated through viability or proliferation assays, do not account for the presence or phenotype of the very rare population of cells that survives treatment below the limit of detection of the assay. It has been assumed by most researchers that observed PGCCs do not survive and die due to mitotic catastrophe subsequent to multipolar cell division. Indeed, the only way to appreciate the presence of PGCCs in tissue culture at all is to directly observe them through microscopy.

There is a small body of literature specifically related to PGCCs (74 PubMed listed entries with query: [polyploid giant cancer cells][26–99] versus 3,857,567 with query [cancer]; accessed 05/10/2019; Figure 2), but this literature, taken holistically, makes a compelling case for defining them as the essential keystone cancer species and actuators of tumorigenesis, therapeutic resistance, and recurrence in metastatic disease. Keystone species are relatively few in number, but have a significant impact on the health and composition of the ecosystem. In this case, the survival of keystone cancer species mediates 1) metastatic spread, 2) survival of cancer cells during and after therapeutic insult and 3) clonal expansion to generate a clinically significant tumor mass. In cancer biology terminology, this translates to PGCCs playing critical roles in all 3 capacities: in metastasis, therapeutic resistance, and having stem-like capacity to asymmetrically divide to give rise to a clonal population of cancer cells. Of the 74 publications, 15 address metastasis, 16 discuss therapeutic resistance,

and 20 explore stem-like characteristics (Figure 2). Notably, only 2 publications combine all three essential characteristics under the investigation of PGCCs.

The presence of PGCCs has been described in a multiple cancer types (breast, ovarian, colon, melanoma, lung, pancreas, urinary bladder, renal, thyroid, prostate), but systematic analyses to assess PGCC status with clinical prognostic have not been performed.[43,54,60,92,100–110] For example, PGCCs have been documented in a PCa patient with pT3bN1Mx, Gleason 5+4=9 (Grade group 5) PCa (Figure 3A). At time of radical prostatectomy, the primary tumor and 7/12 lymph nodes were positive for focal regions of pleomorphic giant cells. Alharbi, et al. analyzed a series of 30 cases of PCa patients with PGCC present in the diagnostic specimen collected from 2005 to 2018.[111] Presence of PGCCs in PCa diagnostic specimens indicates aggressive disease and is associated with a rapid disease course and death. Of the men with a new PCa diagnosis with >1 year follow up, 7/19 (37%) were dead at a median of 8 months. 4/7 (57%) men who had a previous history of PCa were dead at a median of 7 months after diagnosis of recurrent PCa. This is in grim contrast to reported PCa-specific mortality of <5% at 2 years and 10% at 4 years for men with similar nonmetastatic PCa diagnoses (Gleason score 9 and 10).[112] Notably, despite their apparent role in mediating aggressive disease, PGCCs typically make up a small minority of the assessed tumor region (5–20%). These striking data highlight the likely role for these keystone PGCCs as actuators of rapid lethal disease progression.

Studies conducted in yeast, drosophila, cancer models, and clinical data suggest that the polyploidy state mediates therapy-resistant phenotypes.[113] PGCCs have been observed emerging in response to a variety of genotoxic stresses, including anti-cancer therapy such as radiation and chemotherapy, as well as tumor microenvironment-simulating hypoxia[36,56,65,73,78,97,114–122]. In addition to simply emerging in response to stressors, there is evidence that PGCCs contribute to overall therapeutic resistance. Cells derived from PGCCs that form upon Cisplatin treatment have increased resistance to cytotoxic drugs.[78] There is also evidence in castration-resistant prostate cancer (CRPCa) that PGCCs drive resistance to taxane-based chemotherapy.[65,73,117–119,122] The mechanism of this multi-therapy resistance phenotype remains unknown. One hypothesis is that that PGCCs enter a protective and reversible state of therapeutic-induced-senescence, allowing them to survive therapy and later reenter cell cycle to form to daughter cells.[123] Studies have shown that PGCCs express a stem-like phenotype (e.g., expression of self-renewal markers).[124,125] Moreover, there is strong evidence that PGCCs can asymmetrically divide to give rise to daughter cells of typical size and ploidy (Figure 3B).[65,97,122] PGCCs have been shown to re-enter the cell cycle and either undergo error prone aberrant mitoses or an error prone process of cell division independent of a mitotic spindle that uses budding or bursting called amitosis or neosis.[80,85,93,126,127] This stem-like phenotype of asymmetric division gives PGCCs the capacity to generate a clinically evident metastasis of majority non-PGCC cells. In addition, there is recent data that PGCCs that form in response to hypoxia, such as would be found in the primary tumor microenvironment, and in response to therapy have increased metastatic potential, including increased mesenchymal phenotype as well as enhanced migration and invasion.[42,97,128]

As the field of PGCC research grows, it is important to set a definition of the cell type of interest. Pathologists have borrowed language from Virchow, describing regions of "polymorphous giant cells," and there are other reports of "osteoclast-like cells" in tumor sections, describing multinucleated cancer cells. PGCCs have two phenotypic defining characteristics: 1) polyploidy (though not necessarily multinucleation) and 2) relatively large size. Most solid tumors and cancer cell lines are aneuploid (i.e., have an abnormal number of chromosomes or segments of chromosomes). Polyploidy describes a multiple of the baseline set of chromosomes that does not have an upper limit (e.g., 4n, 6n, or 16n). In the case of polyploidy observed in an aneuploid cancer cell line, therefore, it would be a multiple of that "aneuploid n." Importantly, polyploidy does not require multinucleation and can simply present as a single large nucleus, though cells with multiple nuclei are likely polyploid. The other defining phenotypic characteristic of PGCCs is their "giant" morphology (Figure 3A–B). PGCCs are physically and visually larger than their surrounding sister cells, not just with elevated genomic content, but also cytoplasmic area. Further research is needed to assess if size or deformability of PGCCs is biologically significant.

In order to understand and eventually target PGCCs, it will be important to both study PGCCs in isolation as well as in their native ecosystem, especially to appreciate the initial emergence of PGCCs in the primary tumor ecosystem. While there are not currently any biomarkers for PGCCs, either for monitoring *in vivo* or for isolation, this should be an area for future research. The most commonly used assays to study PGCCs rely on microscopy of *in vitro* cultures in order to capture essential PGCC events, such as formation and cell division. Currently, there are no commonly adopted high-throughput methods to isolate a pure population of PGCCs. One of the most common methods to quantify (and in rare cases to isolate) PGCCs in a population is flow-cytometry using standard cell cycle analysis to isolate relative >4n cells (e.g., with 7AAD or other DNA stain. Addition of Cyclin-B1 staining can be used discriminate the G_2/M diploid cells that have undergone S phase and so have 4n genomic content. Overall, however, such flow-cytometry methods drastically reduce viability and are impractical due to the long assay time. Theoretically, researchers may be able to take advantage of the relative size difference of PGCCs compared to the other cancer cells in the population using size-exclusion techniques such as have been developed for circulating tumor cell research, but such a method has not been widely adopted. Importantly, conventional laboratory assays designed to assess efficacy of anti-cancer therapy (e.g., viability or proliferation assays taken days after treatment of cells *in vitro* or tumor recurrence measured weeks after treatment of tumor-bearing animals *in vivo*) do not account for the rare population of surviving PGCCs that exist below the limit of detection until they reenter cell cycle.[62] The majority of modern methods to count and assess cell viability do not require the investigator to actually look at the remaining population. Observing a population of PC-3 cells 72 hours after treatment with a LD90 dose of docetaxel reveals that the majority of cells are PGCCs (Figure 4) (personal communication, Amend and Pienta). Assessing PGCCs *in vivo*, either in histological sections or in liquid biopsies, has its own challenges. It is difficult to assess cell membranes from a typical H&E stain, making polyploidy difficult to ascertain, though focal regions of majority PGCC phenotype (called "polymorphous giant cells" or "osteoclast-like cells") have been reported. Addition of a membrane stain followed by careful evaluation by a skilled pathologist would provide an

opportunity for assessing single or rare PGCC status in patient samples. The presence of PGCCs in the circulation has not been systematically assessed. In liquid biopsy research, it is important to carefully review the algorithm requirements. Many such automated counting systems define a cell as one with a single and/or small nucleus, and so would automatically eliminate any multinucleated or large-nucleated cell from analysis, including possible PGCCs.

While we and others have highlighted the likely role for PGCCs in mediating disease resistance, there are no currently available therapies to specifically target these cells. Indeed, PGCCs emerge in response to all tested standard-of-care therapeutics. As they have a unique phenotype, however, PGCCs may have unique vulnerabilities. For instance, it is clear that in order to divide, it is likely that PGCCs may have to use different cell division machinery than non-polyploid cells (e.g., microtubule organizing center [MTOC] assembly). With such elevated DNA content, it is likely that cell cycle checkpoints may also represent a viable therapeutic target. As discussed above, there is evidence that PGCCs exit the cell cycle and enter a quiescent state. Restraining the cells in that G_0 state may represent a way, not to eliminate the PGCCs, but to prevent the lethal tumor burden that arises when the PGCCs reenter cell cycle. Clearly, this is a critical area of further research – to define therapeutic targets and determine optimal delivery to eliminate the keystone PGCCs from a tumor. Importantly, the PGCCs represent a minority population of the tumor burden, any anti-PGCC therapy can be used in combination with current standard-of-care that will eliminate the bulk of the tumor population.

Metastatic cancer remains incurable because a subset of cells within a tumor has intrinsic or develops resistance to anti-cancer therapy. While standard-of-care hormone therapy, chemotherapy, or radiation may reduce overall tumor size, only a single or a few cancer cell “seeds” are required to mediate metastasis and therapy resistance. These keystone cancer cells, while few in number, exert a large effect on the tumor ecosystem. PGCCs have been observed for more than a century since the first descriptions of cancer cells by Müller and Virchow. It is clear from the limited available clinical data that presence of PGCCs in localized or recurrent prostate cancer indicates a dismal prognosis. The current PGCC literature, though limited, suggests that this distinctive phenotype of cancer cell can 1) initiate the metastatic cascade, 2) survive “lethal” doses of therapeutic, and 3) asymmetrically divide to generate typical cancer cells with increased resistance to different classes of anti-cancer therapy. To cure metastasis, the PGCCs actuating metastasis, therapy resistance, and tumor outgrowth must be eliminated.

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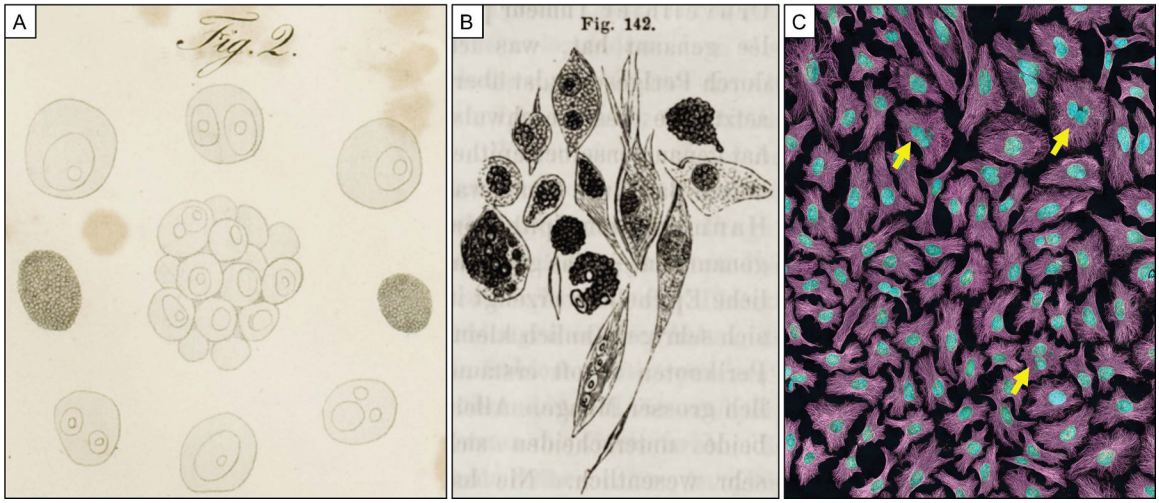
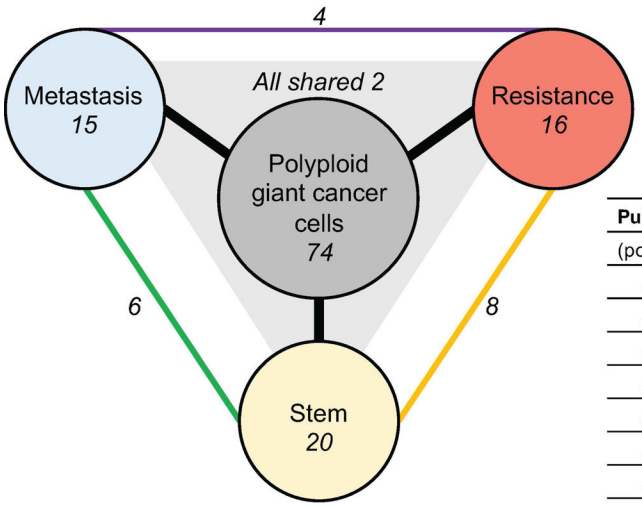


Figure 1. Historical evidence of polyploid giant cancer cells.

(A) Illustration (Plate II, Fig 2) from *Ueber den feinern Bau und die Formen der krankhaften Geschwülste (translated: On the Finer Structure and Form of Morbid Tumors) by* Johannes Müller, 1838; Caption translates "*Cell spheres with germ cells and the nuclei of the germ cells…of Carcinoma reticulare."* (Public domain, CC BY-SA 4.0) (B) Illustration (Fig 142) from Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebenlehre (translated: *Cellular pathology as based upon Physiological and Pathological Histology*) by Rudolph Virchow, 1858 (translated by Frank Chance); Caption: "Various, polymorphous cancer-cells…two with multiplication of nuclei. 300 diameters." (Public domain, CC BY-NC 4.0) (C) PGCCs in HeLA cell culture indicated by arrows. Multiphoton fluorescence image: cytoskeletal microtubules, magenta; DNA, cyan. (Image by NIH, public domain, CC-PD-Mark)



PubMed query	entries
(polyploid giant cancer cells)	74
AND (metastasis)	15
AND (stem)	20
AND (resistance)	16
AND (metastasis) AND (resistance)	4
AND (metastasis) AND (stem)	6
AND (resistance) AND (stem)	8
AND (metastasis) AND (resistance) AND (stem)	2

Figure 2. PubMed queries of the polyploid giant cancer cell literature.

PubMed-listed entries of indicated queries accessed on 05/10/2019. Entries that are listed with multiple search terms are indicated by connecting edges.

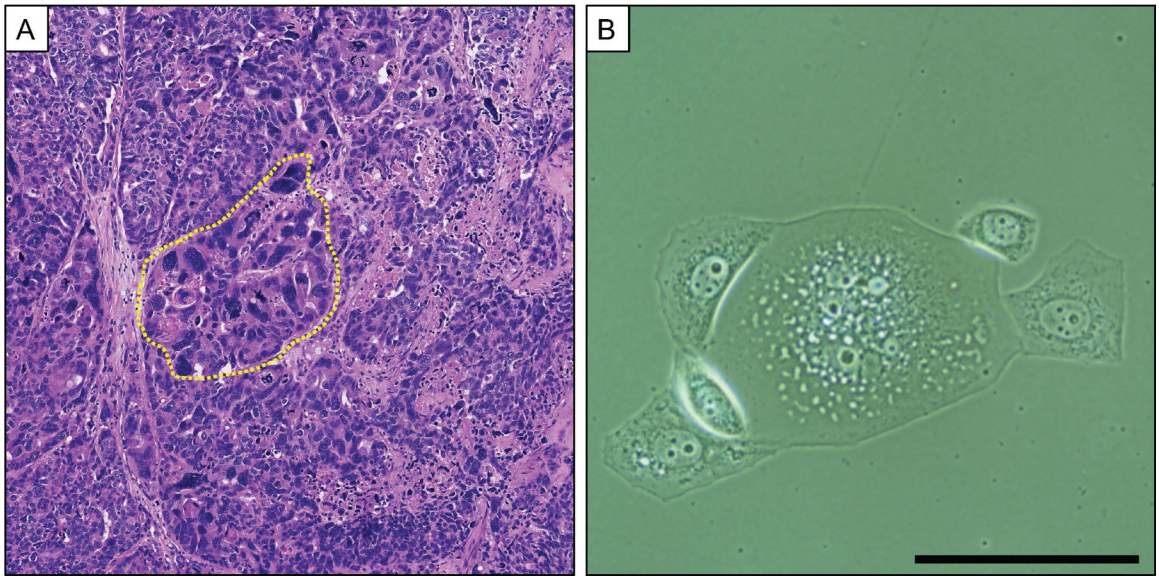


Figure 3. Polyploid giant cancer cells in prostate cancer *in vivo* and *in vitro*.

(A) H&E image of a lymph node prostate cancer metastasis with PGCCs (one region indicated by yellow border). (B) Phase image of a PC3 PGCC undergoing asymmetric division to form mononuclear and typical-sized daughter cells. PC3 cells were cultured with 10nM Docetaxel for 3 days followed by 4 days in Docetaxel-free media. (scale = 200 um)

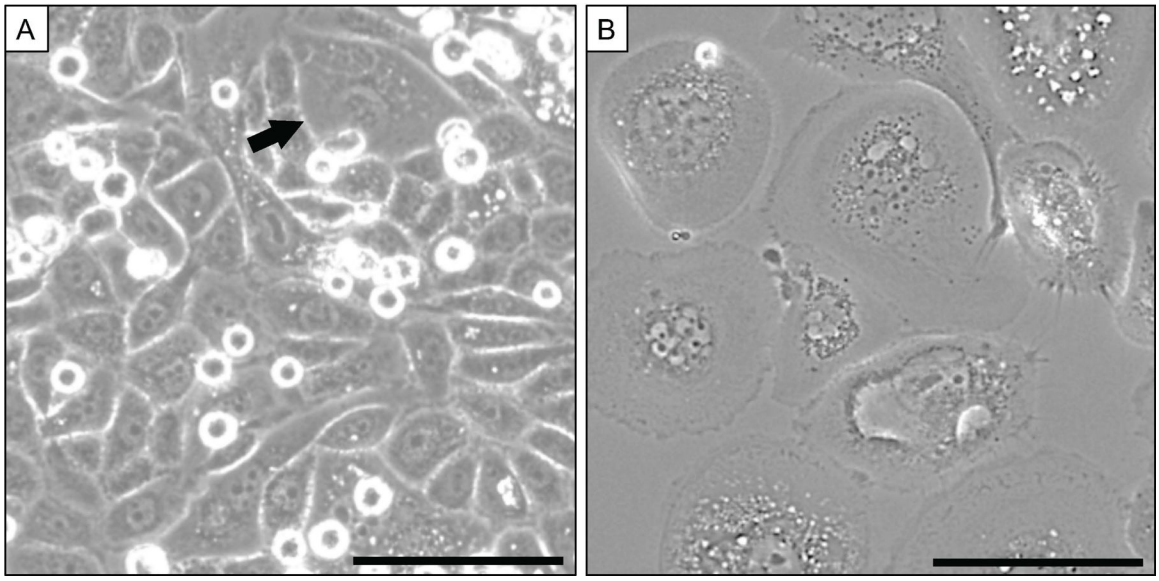


Figure 4. Polyploid giant cancer cells are the majority population following docetaxel treatment *in vitro*.

(A) PC3 cell culture at baseline contains rare PGCCs (arrow). (B) After treatment for 72 hours with LD90 docetaxel, PGCCs are the dominant population and virtually no non-PGCC cells remain. (Phase contrast; scale = 100 um)