Using space-based investigations to inform cancer research on Earth

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Abstract | Experiments conducted in the microgravity environment of space are not typically at the forefront of the mind of a cancer biologist. However, space provides physical conditions that are not achievable on Earth, as well as conditions that can be exploited to study mechanisms and pathways that control cell growth and function. Over the past four decades, studies have shown how exposure to microgravity alters biological processes that may be relevant to cancer. In this Review, we explore the influence of microgravity on cell biology, focusing on tumour cells grown in space together with work carried out using models in ground-based investigations.

Nuclear force
The force holding together subatomic particles of the nucleus.

Electromagnetic force
The force associated with electric and magnetic fields.

Microgravity
Conditions of reduced gravity experienced specifically in the space environment.

Standard gravity
The natural force of attraction exerted by Earth on objects at or near its surface.

Low Earth orbit
A circular orbit extending to approximately 1,200 miles above the Earth's surface.

Gravity-dependent convection
Movement of fluid or gas affected by gravity.

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Four fundamental forces are at work in our universe: weak nuclear force, strong nuclear force, electromagnetic force and gravitational force[1]. Gravity is the attractive force between all matter and is the most familiar force in everyday life — so familiar that it is rarely considered as an experimental parameter in biological studies. In space, however, the force of gravity is diminished, resulting in microgravity. In the microgravity environment of space, the apparent weight of an object or a system is substantially reduced compared with its actual weight in standard gravity (1 g) on the surface of the Earth, and local acceleration experienced in space is a small proportion of that felt in 1 g (REF. 2). For spacecraft in low Earth orbit, vehicles can travel in a state of free fall at speeds in excess of 17,000 miles per hour, and gravitational force ranges from 10^{-4} g to 10^{-6} g (REF. 3). For example, aboard the International Space Station (ISS), a full orbit around the Earth is completed in approximately 90 minutes.

In the 1970s, humans began spending extended periods of time aboard orbiting spacecraft, thus paving the way for conducting biological experiments in space (TIMELIME). As fundamental investigations began in the space environment, it became evident that biological properties change as gravitational force is diminished, underscoring the relationship between physical force and biological function. Cells exposed to microgravity can be profoundly affected by the physical changes that occur in this unique environment, which include the loss of gravity-dependent convection, negligible hydrodynamic shear and lack of sedimentation[1,4,5]. This results in the ability of cells to colocate, coalesce and form complex multicellular aggregates and organoids, which can be sustained for days or months in microgravity[3,6].

It is important to note that, for the purposes of this Review, the term microgravity applies specifically to the conditions of reduced gravity that are experienced in the space environment. Studies conducted in space have been complemented by the use of relevant cell culture paradigms that provide culture conditions akin to those observed in the microgravity environment, including reduced fluid shear, cellular colocation, optimized suspension and three-dimensional (3D) growth. Combining the insight gained from space-based investigations together with data from on-ground culture models that are capable of sustaining the culture conditions observed in space could afford a new approach to the study of cancer.

Early cell-based studies in microgravity

Initial investigations of the effects of microgravity on human cells were carried out during the US Skylab Program in the early 1970s. These missions were carried out to gain insight into physiological and biomedical changes that occur during extended exposure to microgravity in low Earth orbit, ranging from 28 days to 84 days. Data from Skylab demonstrated that living biological systems, from humans to single cells, can readily adapt and thrive in the space environment. WI-38 human embryonic lung cells cultured for up to 28 days during Skylab III showed no significant changes in growth rate, chromosome banding, mitotic index or cell cycle compared with ground control cells[7,8]. However, differences were observed in spent media constituents between flight and ground cultures, including increased glucose concentrations in flight cultures, which suggests altered metabolism in microgravity. Examination

At a glance

- In the microgravity environment of space, cells assemble into multicellular three-dimensional constructs.
- Reduced gravitational force has been shown to have far-ranging effects on cell growth and function, including effects on gene expression, the production of soluble factors, cell signalling and cytoskeletal organization.
- Suspension-based cell culture can be achieved using the rotating wall bioreactor, clinostat, random positioning machine and magnetic levitation. These models provide certain conditions that are observed during culture in microgravity, including lack of sedimentation, reduced fluid shear, optimized cellular colocation and three-dimensional growth.
- Research approaches derived from space-based investigations may be applicable to advance our knowledge of tumour biology, as well as inform the development of new anticancer technologies and therapeutic strategies.

of peripheral red blood cells from Skylab crew verified previous spaceflight data demonstrating reduced red blood cell mass on return from orbit[9] and also revealed substantial alterations in the distribution of erythrocyte shape, from discoid to echinocytic, with increased time in space[10]. Interestingly, all erythrocyte shape changes were reversed within several hours of landing. Lymphocyte counts remained unchanged in Skylab astronauts; numbers of neutrophils were increased at landing, but returned to baseline within several days. Lymphocytes displayed varying amounts of surface microvilli[10], and decreased mitogen-induced blastogenesis has been observed[9–12]. This work was the foundation for subsequent experiments that have further examined how reduced gravity affects the growth and function of both normal cells and cancer cells.

Relevant cell culture models on Earth

Space-based research has been part of the paradigm shift in the field of cell biology, for which new tools have been created for culturing cells in three dimensions. It is now well understood that 3D growth environments that facilitate unrestricted cell–cell interactions are important for defining the biology of cancer cells and tissue, including tumour formation, the tumour microenvironment and tumour progression[13,14]. Early studies by Emerman and collaborators[15] demonstrated that the tissue-like structure of mammary epithelial cells could be maintained in 3D-type floating collagen gels. Work by Bissell and Brugge on breast carcinoma has shown the importance of 3D architecture for creating biologically representative *in vitro* cancer models[16–20]. Nederman, Carlsson and colleagues showed that human osteosarcoma, glioma and thyroid carcinoma cells grown as multicellular spheroids *in vitro* exhibit functional and structural characteristics akin to growth occurring *in vivo*, including the expression of complex extracellular matrix and lowered pH within interior portions of the spheroid[21–25]. Acker[26] and Kunz-Schughart *et al.*[27] have suggested that 3D spheroids cultivated *in vitro* represent ideal systems for examining aspects of the tumour environment that are observed *in vivo*. In order to achieve an accurate representation of the tumour microenvironment, it is necessary that 3D multicellular constructs generated *in vitro* exhibit properties that are observed *in vivo*, including the production of extracellular matrix, cell polarization and spatially unrestricted cell–cell interaction[16–27]. To date, monolayer culture is still one of the most commonly used methods for growing cells, where gravity prevails as a dominant force. When anchorage-dependent cells are transferred to a culture flask, gravity drives them to the bottom of the container where they adhere, flatten and divide as a 2D monolayer. In contrast to growth *in vivo*, monolayer culture yields artificial conditions in which cells interact with a stiff, flat surface and have limited cell–cell interconnectedness. Therefore, it is important to use *in vitro* methods that support biologically representative models of the *in vivo* 3D growth environment.

The RWV bioreactor and clinostat. The ability to achieve unrestricted 3D growth in suspension on Earth, as occurs in microgravity, is a problem that was addressed by NASA (National Aeronautics and Space Administration) through the development of the rotating wall vessel (RWV) bioreactor[28–31]. The RWV is a horizontally rotating vessel with no internal mechanical agitator. A central silicone membrane is present in the RWV that delivers oxygen via diffusion, avoiding the production of bubbles that are disruptive to the growing cells. The vessel is completely filled with culture media and thus has no air–liquid interface (FIG. 1). Because there are no internal moving parts, the vessel provides a culture environment that is characterized by low shear and low turbulence. Within the RWV, adherent cells are grown on microcarrier beads to provide a solid support; the cells readily attach and cover the surface of the microcarriers. With continued growth, multiple cell-covered beads coalesce, undergo cellular bridging and generate high-density, 3D aggregate structures under conditions of optimized suspension and uniform mixing to allow for the effective transfer of nutrients and metabolic waste[29–31]. As the RWV rotates, culture fluid achieves near laminar flow and the cellular aggregates present in the fluid are in a state of free fall, but never reach the bottom of the vessel owing to the constant rotation of the RWV. The RWV was originally designed to replicate certain conditions that occur in the reduced gravity environment of space, including the lack of sedimentation, substantially reduced fluid shear and optimized cellular colocalization; the physics and engineering principles of RWV culture are described in detail elsewhere[5,28,32].

The RWV has been considered as a specialized form of clinostat, as both culture devices operate by the process of clinorotation; that is, rotation about their horizontal axis[32,33]. The slow-rotating clinostat has a long history of use in plant biology; more recently, the fast-rotating clinostat has been applied to microbial culture and cell culture[32,34,35]. Like the RWV, the clinostat operates as a completely fluid-filled vessel and, via horizontal rotation, facilitates the suspension of cells as they fall through the culture medium. Although the RWV and the clinostat both supply near-laminar flow, the RWV provides membrane oxygenation to the growing culture, which is not a feature of the clinostat. In the clinostat, cells may be cultured in a monolayer on glass slides, which is not a feature of the RWV. Importantly, neither culture vessel

Hydrodynamic shear
Stress arising in a fluid that is a function of the fluid velocity gradient and viscosity.

Sedimentation
The settling of solid material from a state of suspension.

Discoid
Disc-like shape of normal red blood cells.

Echinocytic
Abnormally shaped red blood cells exhibiting blunt spicule protrusions.

Spheroids
Three-dimensional multicellular clusters or aggregates.

Turbulence
Disordered motion in a fluid yielding disrupted and irregular flow.

Laminar flow
Fluid flow occurring in layers.

Clinostat
A horizontally rotating culture device.

Clinorotation
Rotation of a culture vessel about its horizontal axis.

Membrane oxygenation
Oxygen delivered to cells in a culture vessel via a gas-permeable membrane.

Gravitational vector
Unidirectional downward pull of the force of gravity.

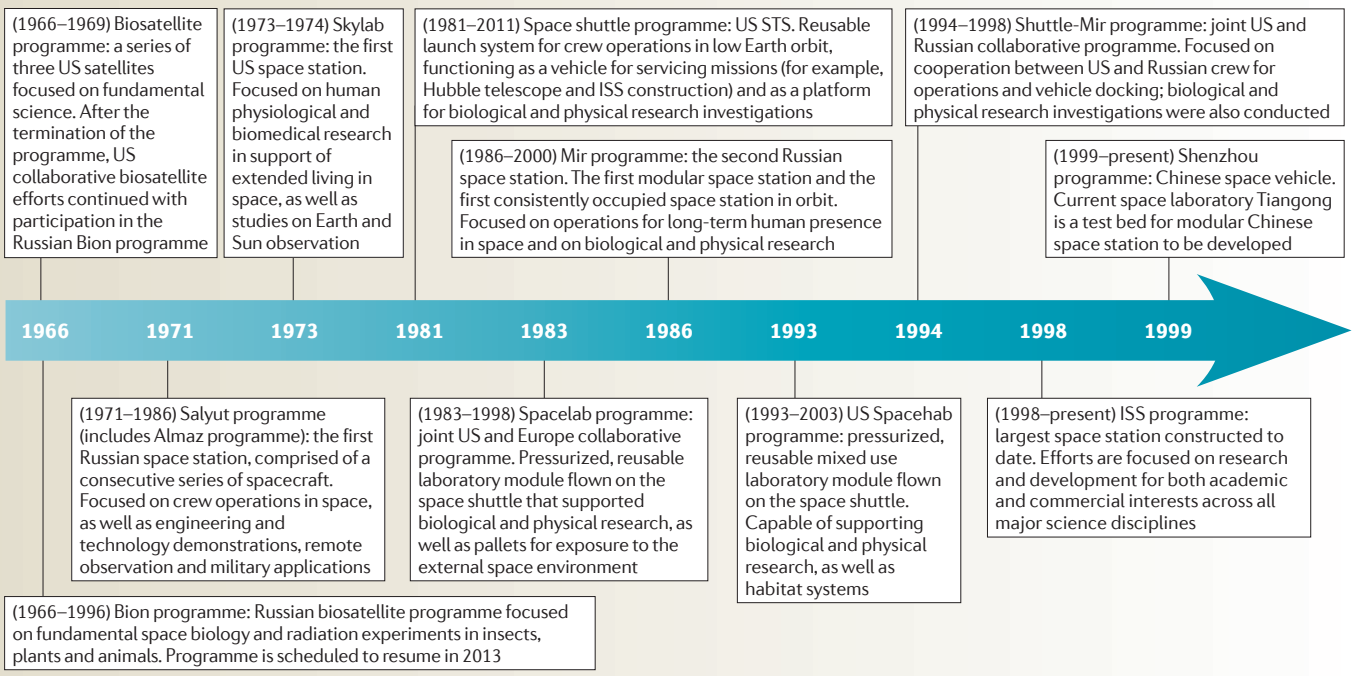
removes the force of gravity; the continuous rotation of the vessels yields a constant randomization of the gravitational vector, but gravity remains present[5,32,35].

Both the RWV and the clinostat have been used to prepare for cell-based experiments to be conducted in space, as growth conditions in the two systems permit useful modelling for experimental design and flight payload logistics. However, RWV technology has proved to have broad applications for the generation of 3D architecture for numerous cell types, including cancer cells. This cultivation technique allows for the production of large batches (10 ml to 500 ml volumes) of 3D multicellular aggregates, and supports the ability to obtain serial samples without the disruption of the culture as a whole, which facilitates cell expansion and the long-term study of continued development of 3D cellular architecture. Growth in the RWV has supported the development of models of breast cancer[36–38], cervical cancer[39], colon cancer[40–42], hepatocellular carcinoma[43,44], neuroblastoma[45], melanoma[46–48], ovarian cancer[49,50] and prostate cancer[51–55]. Clinostat culture has supported the growth of astrocytoma[56], epidermoid carcinoma[57–59], Leydig tumour[60], melanoma[61], neuroblastoma[62] and osteosarcoma cells[63–65]. Key findings from these studies are discussed in more detail below.

The RPM. The random positioning machine (RPM; also known as the 3D clinostat) can support certain conditions of the space microgravity environment, including the lack of sedimentation to facilitate cell colocation and growth of multicellular spheroids[66–71]. As opposed to the RWV in which cells are cultured entirely in suspension, in the RPM a tissue culture flask containing a subconfluent monolayer of cells is affixed to the centre of a platform in an interconnected framework that is comprised of two perpendicular arms that rotate independently of each other, to create continuous random directional adjustment of the culture flask (FIG. 2). The flask is filled completely with media and aeration occurs via a gas-permeable cap. During growth in the RPM, cells can detach from the surface of the flask and form multicellular spheroids, hence incubation in the RPM yields cell growth in suspension, as well as cells remaining adhered in 2D culture. In the RPM, the gravity vector is constantly reoriented, as occurs in clinorotation, but this occurs with increased directional randomization in a manner such that no prevailing orientation occurs; the physics and engineering principles of RPM culture have been described in detail elsewhere[72–75]. The device may be operated either as a clinostat, in which only one of the two supporting frames rotates, or in 3D mode whereby both frames rotate. As described above for the clinostat and the RWV, continuous rotation of the RPM provides constant randomization of the gravity vector, making this device a useful adjunct to prepare for spaceflight studies. The RPM has been used to develop culture models of malignant glioma[76], thyroid carcinoma[66–71] and leukaemic cells[77,78]; the key findings from which are discussed below.

Magnetic levitation. Although various techniques are available to generate 3D architecture *in vitro*, including extracellular matrix protein gels[18,19,79], liquid overlay using thin layer agarose[21–25] and polymeric scaffolds[19,80–83], these approaches do not maintain cells in suspension, as is the case in the RWV, clinostat and

Timeline | **Experimenting in space***



ISS, International Space Station; STS, Space Transportation System.*The years refer to the dates in which the programme was in use in space.

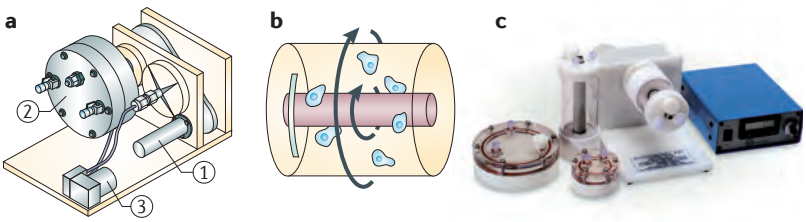


Figure 1 | **RWV bioreactor 3D cell culture.** **a** | A schematic representation of the rotating wall vessel (RWV) illustrating the motor (1), which powers a belt that rotates the culture vessel (2) about its horizontal axis is shown. Incubator air is pumped in (3), filtered and delivered to the growing culture. Sample ports on the vessel exterior facilitate media exchange and sample acquisition. **b** | A representation of three-dimensional (3D) constructs rotating within the vessel, about the horizontal axis is shown. **c** | A variety of vessel types permit culture volumes ranging from 10 ml to 500 ml. Image in Part **c** courtesy of Synthecon, Incorporated, USA.

RPM. The maintenance of 3D constructs in suspension permits spatially unrestricted growth, and facilitates the transfer of nutrients to the growing culture. An alternative means to counteract gravitational force and to achieve 3D growth in suspension is via magnetic levitation[14,84,85]. Magnetic levitation has been used to evaluate gravisensing (that is, gravity sensing) mechanisms in biological systems[86–88] and has been applied to facilitate insight into the effects that may occur in gravity conditions of Lunar and Martian environments[89]. In magnetic levitation culture, the suspension of cells occurs at the point at which gravitational force is counterbalanced by magnetic force. A system to levitate cells in 3D has been developed in which a magnetic nanoparticle assembly is electrostatically adhered to cells, allowing them to lift off the surface of the culture dish in the presence of a static magnetic field, which is provided by a small neodymium magnet (FIG. 3). The cells coalesce adjacent to the air–media interface where they quickly assemble into 3D multicellular constructs. Magnetic nanoparticle levitation was originally applied as a tissue culture model to facilitate the generation of large 3D constructs of human glioblastoma cells (FIG. 3). These aggregates exhibited high-fidelity cellular organization, compared with tumour cell xenografts, and supported the co-culture of the glioblastoma cells with normal human astrocytes to establish an *in vitro* 3D suspension model of tumour cell invasion[84]. Using magnets of different geometries, varying shapes of 3D constructs can be generated, thus facilitating the investigation of the influence of spatial configuration in constructs comprised of multiple cell types, including malignant cells and normal cells. Moreover, using different magnet geometries has enabled the adaptation of this methodology to multiwell plate configurations[90,91] (FIG. 3f). This technique has been applied to 3D culture of breast cancer, hepatoma, kidney cancer, lung adenocarcinoma, melanoma, osteosarcoma and prostate cancer (G.R.S., unpublished observations). The use of magnetic nanoparticle levitation in multiwell plates is compatible with standard and high-throughput labware, and can support the adoption of high-fidelity 3D models for cancer drug screening[92]. Magnetic levitation of mammalian cells has also been accomplished using a warm bore superconductive magnet, although this tissue culture apparatus requires cryogenic temperatures (liquid helium) for cooling superconductive coils, and can subject cells to magnetic fields more than an order of magnitude higher than those generated with permanent magnets[93,94].

Warm bore superconductive magnet
A strong field (~100 T per m gradient) magnet, similar to a high-field nuclear magnetic resonance spectroscopic magnet.

Advantages and constraints of the cell culture models. The connection between structure and function can be examined using 3D cell culture. The RWV and the magnetic levitation model can support the generation of large 3D constructs with complex cellular architecture and structural characteristics that are biologically representative of *in vivo*-like growth (FIG. 4). However, the manipulation of cellular responsiveness on both the single-cell and the multicellular levels may yield information about how the physical interactions of cancer cells with each other, and with their surrounding environment, are affected by architecture. In this regard, the clinostat can support suspension or monolayer culture and the RPM can support the transition from 2D monolayer to 3D spheroid culture during continuous randomized rotation, thus it has been suggested that the RPM may facilitate the study of cellular events that occur during this shift[71]. However, neither the RWV bioreactor, the clinostat, the RPM nor magnetic levitation removes the force of gravity from the system[5,32,35]. Rather, the models may provide an environment that reflects some of the conditions that are observed in the microgravity environment of space, including the lack of sedimentation, reduced fluid shear, cellular colocation and 3D multicellular growth.

Each of the model systems offers operational advantages and constraints that can be used to approach experimental problems (BOX 1). For example, large constructs are difficult to maintain in suspension in the RWV, hence 3D growth can become size limited. However, because a small amount of fluid shear is present in the RWV, this system can be used to study the influence of shear stress on cancer cell constructs, as can the RPM. Fluid shear has an important role not only in metastasis[95–97], but also in tumour cell shape and cytoskeletal architecture[98,99]. Another consequence of 3D architecture in these models is the development of small areas of necrosis during continued growth. The presence of focal necrosis in the constructs might initially be perceived as a disadvantage of the culture models. However, the regions of hypoxia surrounding necrotic foci can lead to the establishment of microenvironments that are characterized by low tumour cell metabolic activity, heightened drug resistance and epithelial-to-mesenchymal transition, all of which are factors that influence tumour progression and metastasis[100–102]. The ability to model such tumour microenvironments *in vitro* could be an important advantage to the study of tumour progression and chemoresistance. Small, rapidly proliferating multicellular constructs that do not contain necrosis may simulate actively growing areas in a tumour that are responsive to chemotherapeutic agents, where oxygenation and nutrients are non-limiting. By contrast, large constructs

exhibiting areas of necrosis may model bulky tumours that have regions of low proliferative activity and increased drug resistance. In fact, a similar approach has been undertaken in spheroid models of colorectal cancer[103] and pancreatic cancer[104], in which diminished responsiveness to antitumour agents was observed as a function of multicellular architecture, reduced proliferation and microregions of hypoxia. Multicellular drug resistance — that is, chemoresistance owing to 3D structure — is not a new concept and has been studied for the past three decades[22,24,26,27,105–108]. This body of work has shown that tumour cells cultured as 3D aggregates exhibit significantly increased drug resistance compared with 2D growth in monolayer culture.

Cellular alterations in microgravity

Immune cell changes. Investigations of the effects of microgravity on immune cells carried out over the past three decades on the Space Shuttle, the ISS and on Russian vehicles have yielded interesting, and conflicting, data. Leukocytes from rodents flown in space exhibited suppressed lymphocyte mitogenesis, inhibited natural killer cell cytotoxic activity, reduced cytokine production and alterations in the function and proportions of T cell subsets[109–113]. By contrast, other studies have demonstrated increased cytokine production by spleen and thymus cells of space-flown rodents, and by immune cells cultured in microgravity, including interleukin-1 (IL-1), IL-3 and IL-6; tumour necrosis factor (TNF); and interferon-α (IFNα) and IFNγ[114,115]. Despite the potential for the suppressed responsiveness of various facets of the immune system, mortality due to cancer in astronauts is not increased relative to the general population[116]. Moreover, the Longitudinal Study of Astronaut Health found that the incidence of cancer among astronauts is reduced relative to the US National Cancer Institute's Surveillance, Epidemiology and End Results database; prostate cancer was the most prevalent malignancy noted in the study[117].

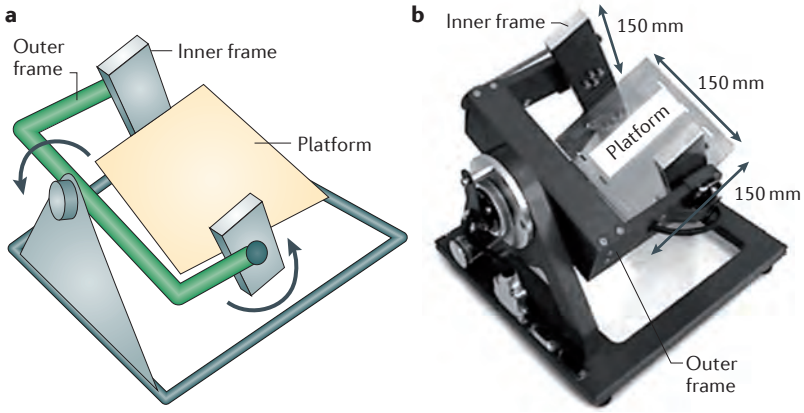


Figure 2 | **The RPM.** **a** | A schematic representation of the random positioning machine (RPM) illustrating the inner and outer frames, which rotate independently of each other (designated by curved arrows) is shown. The culture vessel is mounted in the centre of the platform. **b** | The desktop RPM is composed of two motors that independently control the rotation of the two frames; sizes of the frames and platform are indicated. Part **b** is reproduced, with permission, from REF. 75 © (2005) American Physiological Society.

Alterations in gene expression. Some of the studies mentioned above examined changes in the expression of specific genes, predominantly those encoding cytokines, during spaceflight[114,115]. The effect of microgravity on global gene expression was evaluated in human primary renal cortical epithelial cells[118,119]. The cells were cultured for 6 days on the Space Shuttle and readily grew into 3D aggregates. Microarray analysis of 10,000 genes revealed that the expression of 1,632 genes was altered in microgravity, relative to 1 g controls[118,119]. The changes in expression comprised a number of genes that are not associated with cell stress responses, including decreased levels of Wilms' tumour 1 (*WT1*), significantly increased levels of vitamin D receptor (*VDR*) and villin 1 (*VIL1*), as well as differences in several cytoskeletal genes. These results from Hammond and colleagues[118,119] were the first demonstration of the ability of reduced gravity exposure to elicit broad modulation of cellular gene expression. Scanning electron microscopy demonstrated that the renal cells grown in orbit achieved larger sized 3D aggregates and displayed increased surface microvilli, compared with controls. Microarray analysis of 20,000 genes in Jurkat human leukaemic cells cultured for 2 days on the Space Shuttle, compared with ground controls, revealed differences in the expression of several gene groups, including those involved in cell cycle regulation, apoptosis, tumour suppression, signal transduction and cytoskeletal organization[120]. These results validated previous morphological analyses of space-flown Jurkat cells that showed early microtubule disorganization (4 hours post-launch), with reorganization occurring at 2 days in microgravity; cells exposed to reduced gravity also exhibited increased release of FAS (also known as APO1 and TNFRSF6)[121]. Space-grown CaSki cervical carcinoma cells showed altered gene expression that generally corresponded to changes in genes regulating the cell cycle, cell morphology, apoptosis and signal transduction[122,123]. Of note, Jurkat cells grown in microgravity also demonstrated enhanced expression of plectin, which studies have suggested may be a clinically useful biomarker for pancreatic ductal adenocarcinoma[124]. Plectin is a key cytoskeletal component, linking together intermediate filaments with actin and microtubules[125]. Evidence indicates that plectin can facilitate tumour cell migration and tissue invasion in head and neck squamous cell carcinoma[126], and in colon carcinoma[127]. Because reduced gravitational force can modulate the expression of genes including plectin, *VDR* and *WT1*, each of which has known roles in cancer, it is tempting to speculate that additional investigations of tumour cells exposed to a microgravity environment may be able to shed light on the regulation of other genes that have been shown to have a potential role in cancer growth and progression.

Effects on cell signalling. Although large-scale functional studies of tumour cells cultured in conditions of reduced gravity have been limited, experiments on cell signalling, secretion of soluble factors and evaluation of cytoskeletal elements have been conducted in space. Growth of U937 myelomonocytic cells for up to 4 days in microgravity

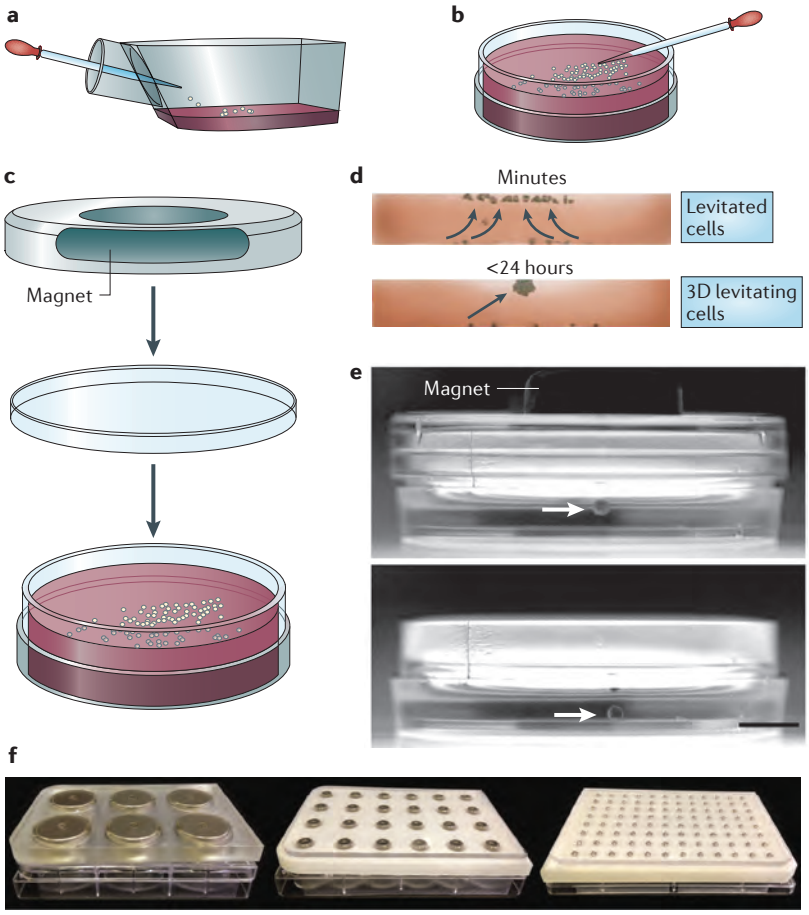


Figure 3 | **Magnetic levitation 3D cell culture.** **a** | Cells in monolayer culture are incubated in culture medium with nanoshuttle-containing magnetic nanoparticles. **b** | After incubation with nanoshuttle, cells are detached and transferred to petri dishes. **c** | Magnetic drive is attached on top of the culture plate (order of application: top, magnetic drive; middle, petri dish cover; bottom, petri dish containing nanoshuttle-treated cells). **d** | Within minutes, cells rise to the air–liquid interface (top; indicated by upward arrows) then self-assemble into a three-dimensional (3D) configuration within 24 hours (bottom; 3D aggregate indicated by arrow). **e** | A 3D culture of human glioblastoma cells grown for 48 hours is shown; 1 mm aggregate is indicated (scale bar = 5 mm). With magnetic drive in place, the aggregate levitates at the air–liquid interface (top). Following removal of magnetic drive, the 3D aggregate settles on the bottom of the petri dish (bottom). **f** | Magnetic nanoparticle levitation systems designed to fit 6-, 24- and 96-well tissue culture plates.

altered both the translocation kinetics and the intracellular localization of protein kinase C (PKC), with similar results observed for space-flown Jurkat cells[128–130]. These studies showed that phorbol ester-stimulated translocation of PKCβII, PKCδ and PKCε was reduced in Jurkat and U937 cells cultured on the Space Shuttle, effects that the authors speculated to be associated with alterations of cellular architecture occurring in conditions of reduced gravity. Following treatment with dihydroxyvitamin D_3 and transforming growth factor-β2 (TGFβ2), MG-63 osteosarcoma cells exhibited significantly reduced expression of mRNA for collagen type I α1 (*COL1A1*), osteocalcin (also known as *BGLAP*) and alkaline phosphatase (ALP) during a 9-day spaceflight study, relative to 1 g controls, effects that correlated with reduced differentiation[131]. HL-60 promyelocytic leukaemia cells exhibited disordered microtubule polymerization when exposed to microgravity for 3 days[132], which coincided with heightened uptake of doxorubicin. These investigators postulated that cytoskeletal rearrangement and the ability of reduced gravity to alter PKC localization could be contributing factors underlying the increased intracellular drug levels that are observed in the HL-60 cells cultured in space.

Microtubule rearrangements were also observed in aggregates of MCF-7 human breast carcinoma cells exposed to microgravity for 2 days, and the cells exhibited prolonged cycling times[133]. Growth of MIP-101 human colon carcinoma cells was altered in space. Ground studies show that, when cultured as a monolayer, these poorly differentiated tumour cells secrete low levels of carcinoembryonic antigen (CEA; also known as CEACAM5) but produce increased levels when implanted into the peritoneum of nude mice[134]. Growth of MIP-101 cells as 3D aggregates for 6 days in microgravity resulted in decreased apoptosis, enhanced differentiation and increased CEA production, relative to monolayer ground controls; changes in the expression of epidermal growth factor receptor (EGFR), TGFα and TGFβ were also observed[42]. When cultured as 3D aggregates in the RWV bioreactor on the ground, MIP-101 cells produced CEA, but exhibited greater apoptosis and reduced proliferation compared with 3D cultures grown in space; MIP-101 cells grown as static (non-rotated) 3D cultures on the ground also showed less apoptosis and increased proliferation relative to rotated cultures, leading Jessup *et al.*[41,42] to postulate that rotation may increase apoptosis in these colon carcinoma cells. Although the effect of growth in space has not been reported, multicellular spheroid cultures of thyroid carcinoma cells grown in the RPM also exhibited increased apoptosis compared with non-rotated cultures[67,68,70,71]. In addition, the thyroid carcinoma cells demonstrated upregulated thyroid-stimulating hormone receptor (TSHR) expression, and decreased secretion of thyroid hormones[66,67], which the authors attributed to a weightless culture environment.

Aggregates of LN1 human mixed mullerian ovarian tumour cells were cultured for 14 days on the ISS (FIG. 5). LN1 cells exposed to microgravity showed reduced expression of vimentin and epithelial membrane antigen, compared with ground controls[135]. Reduced expression of IL-6 and IL-8 was also observed in LN1 cells grown in space, relative to ground controls (J.L.B., unpublished observations). Both of these cytokines have been shown to be associated with the growth of several types of tumours, including ovarian tumours[136–140], and increased expression of IL-6 and IL-8 has been observed in association with multicellular drug resistance in breast cancer cells[141]. Reduced expression of IL-6 and IL-8 was observed in multicellular thyroid carcinoma spheroids grown in the RPM, relative to higher levels of expression of these cytokines reported in the 2D adherent tumour cells in the RPM culture[71]; however, thyroid tumour spheroid expression of IL-6 and IL-8 in space has not been determined. Similarly, the investigation of whether LN1 cells undergo differentiation during time in the reduced gravity of low Earth orbit, as occurs in MIP-101 colon

Histogenesis
Growth and differentiation of cells to form specialized tissue.

carcinoma cells, will require further study in space. LN1 cells have stem cell qualities; when grown as 3D aggregates in the RWV bioreactor, the cells re-express the phenotype of the original mixed lineage tumour from which they were derived[50], characteristics that become lost in monolayer culture[142], thus underscoring the ability of 3D culture conditions to support spatial geometries facilitating the *in vitro* expression of tumour cell properties and the phenotypes exhibited *in vivo*.

One of the most striking demonstrations of tumour growth in microgravity was a study of prostate carcinoma by Chung and colleagues[53] conducted on the Space Shuttle STS-107. Although a tragic accident resulted in the loss of the crew and vehicle, video downlinked during the flight showed that in less than 1 week LNCaP human prostate carcinoma cells co-cultured in space with osteoblasts developed into golf ball-sized organoid structures, whereas aggregates grown in the RWV on the ground only reached diameters of 3–5 mm[53,143]. In earlier work from this group, LNCaP cells co-cultured in the RWV with prostate fibroblasts readily developed into 3D constructs that displayed *in vivo*-like histogenesis and exhibited dihydroxytestosterone-induced growth stimulation and prostate-specific antigen (PSA) production similar to that observed *in vivo*[51]. Similarly, Ingram and colleagues[52] demonstrated that co-culture of PC3 human prostate carcinoma cells with prostate fibroblasts in the RWV also yielded constructs with 3D tissue-like architecture that exhibited upregulated expression of membrane-bound adhesion and extracellular matrix molecules, including the expression of tenascin (TNC) in the fibroblasts. PC3 cell invasion of fibroblast spheroids was also observed. Increased levels of TNC, an extracellular matrix glycoprotein that is expressed in tumour microenvironments, have been shown to correlate with tumour cell invasion and metastasis[144,145]. Sung, Chung and collaborators[54] also reported increased levels of extracellular matrix molecules, including TNC, following 3D co-culture of human prostate cancer cells and bone stroma in the RWV bioreactor. These findings correlated with increased reactive stroma and advanced prostate tumour cell progression in the multicellular constructs, leading these investigators to postulate that spatial orientation due to 3D growth conditions allows the tumour cells and stroma to co-evolve, accelerating prostate cancer cell growth and metastasis.

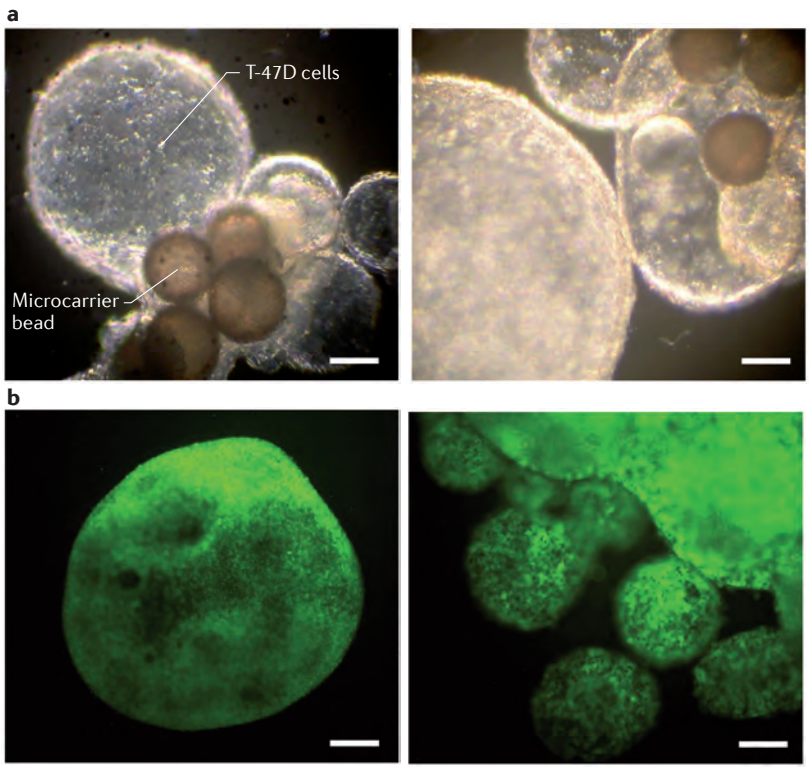


Figure 4 | **3D tumour cell aggregates.** **a** | Photomicrographs of T-47D human breast ductal carcinoma cells grown in the rotating wall vessel (RWV) bioreactor are shown. T-47D cells were cultured on Cytodex-3 microcarrier beads for 42 days. Large aggregates formed that were comprised of multiple layers of cells. The cells initially grew to cover the surface of the bead, after which the 3D outgrowth from the beads readily occurred, and aggregates continued to proliferate in suspension. Scale bar = 200 μm. **b** | Photomicrographs of human glioblastoma cells (labelled with green fluorescent protein) that were levitated for 8 days are shown. Cells coalesced and organized within hours, formed spheroids by 24 hours, and substantially increased in size for the remainder of incubation. Scale bar = 200 μm (left-hand image) and 80 μm (right-hand image).

Cytoskeletal changes in microgravity

A common outcome in nearly all cell types exposed to microgravity is the alteration of cytoskeletal elements: actin, microfilaments and microtubules[146]. Disorganization of basic cellular architecture can affect activities ranging from cell signalling and migration to cell cycling and apoptosis. In a series of investigations that examined microtubule organization *in vitro*, Tabony and collaborators[147,148] hypothesized that both gravity and spatial geometry affect the self-organization of microtubule networks. Their studies demonstrated that tubulin assembles into microtubule structures in a gravity-independent manner. However, the organization of microtubule networks occurs by gravity-dependent reaction–diffusion processes, which are characterized by dynamic growth and the shortening of microtubules; conditions of reduced gravity drive this process to produce alternative orientations relative to those that occur in 1 g. Sample vessel geometry also influenced microtubule self-organization. Space experiments conducted in oval (egg-shaped) containers resulted in reduced microtubule organization compared with 1 g controls, whereas those conducted in rectangular containers exhibited almost no microtubule organization. Similar findings were observed using either clinorotation (as in, the clinostat) or magnetic levitation[149]. These fundamental *in vitro* studies demonstrate that, when the effect of gravity is abridged, processes that were previously controlled or masked by this force may be free to occur in new ways. Cellular biophysical actions that affect tumour cell metastatic capacity, including migration, adhesion and invasion, are influenced by cytoskeletal organization[150–152]. Because microtubules provide architectural support for cellular organization, shape, motility and replication, it is tempting to speculate that disorder caused by exposure to microgravity has the potential to substantially affect cell growth and function. Indeed, compared with 1 g controls, microtubule organizing centres were disordered in Jurkat cells[121] and

Box 1 | **Cell culture paradigms**

Each type of on-ground cell culture method that models some of the conditions of space has characteristics that can be used as an advantage or as a constraint (see the table), but none of the on-ground systems removes the force of gravity from the culture environment. Monolayer culture facilitates good mass transfer of nutrients and metabolic waste via frequent media changes, but does not yield three-dimensional (3D) growth. Culture in the microgravity environment of space supports good 3D growth without fluid shear, but in the absence of mixing, mass transfer can become limiting, resulting in the development of hypoxia and necrosis. The rotating wall vessel (RWV) readily supports 3D growth, but this system produces low levels of fluid shear; fluid shear stress is a function of the 3D construct diameter and the difference in density between the construct and the culture fluid. As 3D constructs become larger in size, the shear stress they experience increases. Fluid shear levels of <0.5 dyn per cm^2 have been reported in the RWV[20] (reviews of RWV operating parameters have been published[20,32]). The random positioning machine (RPM) supports 3D growth following the detachment of cells from monolayers used for culture initiation. Cells grown in the RPM are also exposed to shear stress, with levels of <1.0 dyn per cm^2 reported[75]. Brief periods of mechanical strain may be experienced in RPM culture owing to the force exerted on cell membranes by culture fluid momentum that can occur during sudden directional changes[71,73,75]; a magnitude of <200 microstrains has been reported for cells grown in the RPM[75] (reviews of RPM operating parameters have been published[72,74]). 3D growth occurring by magnetic levitation provides conditions that mirror those observed in space, although low-strength static magnetic field is introduced into the culture system (see REF. 14 for a review of magnetic levitation operating parameters).

Condition	Monolayer culture	Space microgravity	Rotating wall vessel	Random positioning machine	Magnetic levitation
3D growth	–	+	+	+	+
Fluid shear	–	–	+	+	–
Mass transfer of nutrient and waste	Not limiting	Limiting without mixing: compensated by scheduled culture media changes	Not limiting	Not limiting	Limiting without mixing: compensated by scheduled culture media changes

in MCF-7 cells[133] cultured in space; the MCF-7 cells also exhibited a more loosely organized perinuclear cytokeratin network, in conjunction with prolonged time spent in mitosis, compared with 1 g controls. Hence, with the wide use of tubulin-binding agents in the treatment of cancer, including taxanes, vinca alkaloids, colchicine site binders and the newer epothilones, it is conceivable that the investigation of the alterations in cell cytoskeletal organization that occur in conditions of reduced gravity could provide novel insight into the development of potential new antitumour therapeutic agents targeted to microtubules.

Alterations in cell shape. In space, both adherent and non-adherent cell types undergo shape changes and become more rounded[6,146]. The ability of a cell to maintain its shape is the result of a variety of tensional forces that are active within individual cells and between cells interacting with each other and with the extracellular matrix. Tensegrity (also known as tensional integrity) is believed to have an important role in cellular architecture and mechanotransduction, as well as in the response of cells to stress and to their surrounding environment[153,154]. A central tenet of tensegrity is the balance of prestress between tensile cytoskeletal elements that link to structural elements that resist compression; the interplay, or push–pull, between these mechanical forces allows cells to respond to chemical and environmental cues. Mechanical living cell deformation studies have demonstrated that mechanical loads are borne by microtubules, which are balanced by tensile forces in contractile elements of the cytoskeleton[155]. Moreover, the disruption of microtubules yields a transfer of forces to the extracellular matrix, a decrease in cell stiffness and altered cell shape. Evidence also suggests that similar forces that are active on microtubules are integral to the maintenance of nuclear shape and also proposes that the transfer of mechanical stress across the cytoskeleton may link the alterations in cell and nuclear shape that occur during cell spreading and retraction[156,157]. In accordance with the tensegrity model, Ingber[158] has proposed that cells exposed to microgravity may undergo a reduction in prestress that yields altered cellular architecture. Furthermore, the possibility for microgravity-induced anomalies in nuclear architecture to alter gene expression has also been suggested[159].

Shape changes that occur in microgravity can alter cellular adhesion to extracellular matrix, as was observed in rat osteosarcoma (ROS) 17/2.8 cells[160]. Growth of these cells for 9 days in space resulted in a disordered cytoskeleton with significantly decreased focal vinculin expression, which indicated reduced integrin-mediated cell adhesion. Post-mitotic ROS 17/2.8 cells predominantly exhibited these effects; cells in other phases of the cell cycle showed little change relative to 1 g controls. These studies also found no significant differences in cycling times between spaceflight and 1 g cultures. The investigators hypothesized that mechanical forces associated with tensegrity may underlie the vulnerability of the post-mitotic ROS cells to exhibit these effects. Because cell adherence is a necessary step following the completion of mitosis, microgravity-induced alterations of physical factors that produce unbalanced tensional forces between the internal cellular cytoskeleton and the extracellular matrix may lead to reduced adherence as a function of changes in cell architecture; this was shown by osteosarcoma cytoskeletal disarrangement and cell rounding, which remained after space-grown ROS 17/2.8

Tensegrity
Also known as tensional integrity. A biomechanical principle of continuous tension or prestress that imparts stability and integrity in a spatial system and that facilitates responsiveness to environmental cues.

Prestress
Resting tension that provides structural integrity.

cell division was complete. Cytoskeletal disorganization is observed in cancer cells and has been shown to be associated with changes in cellular mechanical properties that contribute to enhanced cell deformability, reduced stiffness and increased metastatic potential[99,161,162].

These studies illustrate that microgravity affects both cell structure and function, which may not only affect cytoskeletal architecture but may also affect overall shape, as well as gene expression and signal transduction (BOX 2). Moreover, although a full discussion is beyond the scope of this Review, space radiation can also affect cell growth and function in microgravity via damage to biomolecules, including nucleic acids and proteins, as well as through the induction of free radicals and cell damage owing to bystander effects[163–165]. Certainly, radiation poses hazards to humans during spaceflight, and studies suggest the use of biomarkers to enable the prediction of risk and to guide the development of appropriate mitigation strategies and biological countermeasures to decrease the effects of exposure[165–168]. Similarly, applying proteomic and genomic approaches to assess cellular pathways and mechanisms that are affected by the space environment in both normal cells and cancer cells may identify targets for new investigations[169,170]. The integration of biological and physical approaches to enhance our understanding of cancer biology is an emerging field, and studies point to the need to apply novel approaches and new technologies to broaden strategies for developing more effective treatment options for this complex disease[98,171].

Space-based technologies for cancer therapy

Aside from the cell-based investigations discussed above, other space-based work has been conducted that is applicable to the study of cancer. Microencapsulation research carried out on the Space Shuttle from 1996 to 1998 evaluated the uptake and encapsulation of fluid-containing particles, imaging materials and anticancer drugs in the ultra-low shear environment of microgravity. These studies paved the way for extended duration experiments conducted on the ISS, capitalizing on microgravity-induced modification of fluid mechanics, and led to the development of a new pulse flow microencapsulation technology for combining immiscible liquids so that the interface of the fluid is dominated by surface tension as opposed to fluid shear[172]. This technology facilitates the encapsulation of particles of dissimilar densities and fluids of different viscosities into complex multilamellar structures. The microencapsulation technology was commercially acquired and tested using a xenograft model of prostate cancer, in which successful tumour treatment was observed when encapsulated 5-fluorouracil was administered alone or in combination with cryotherapy[173,174]. These studies used the microencapsulation technology developed in space for the development of new drug delivery systems administered on Earth.

Light technology studies originally conducted on plants cultivated in space were subsequently determined to be applicable to cancer therapy on Earth. Early work on the development of life support systems to facilitate long-term space exploration examined

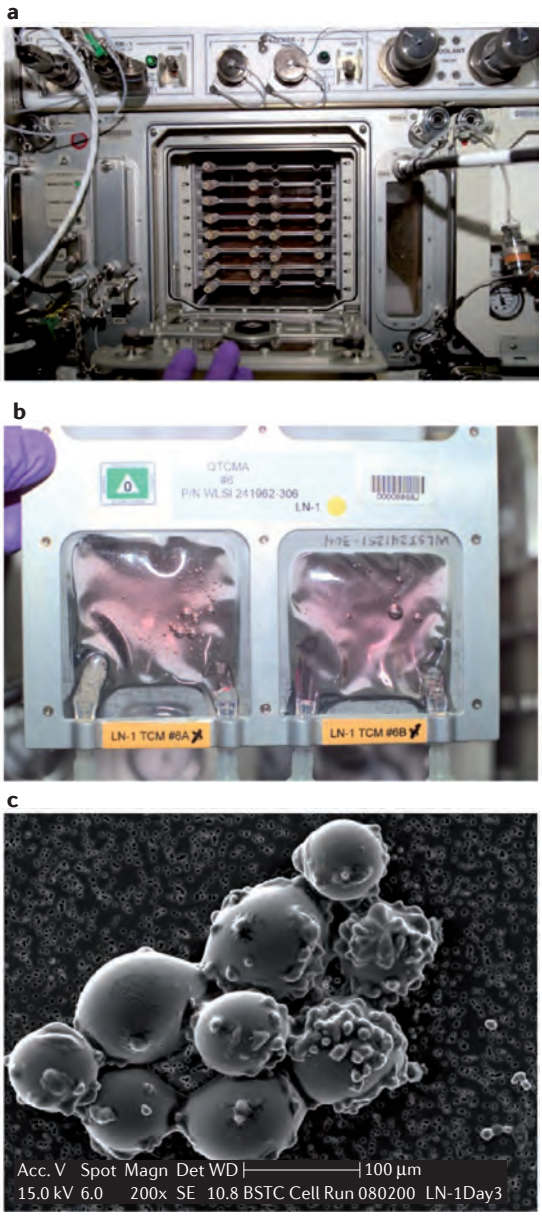
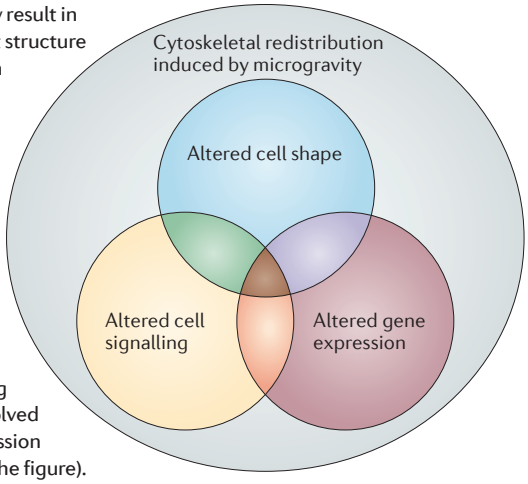


Figure 5 | **Culture of LN1 human mixed mullerian ovarian tumour cells aboard the ISS. a** | Cells were grown for 14 days in the Biotechnology Specimen Temperature Controller (BSTC) hardware, resident on the International Space Station (ISS). The BSTC is a self-contained incubator that supports the growth of up to 32 modules that contain cells. **b** | Tumour cells were grown in gas-permeable tissue culture bags, housed in modular racks in the BSTC hardware. **c** | Scanning electron micrograph showing day 3 cultures of LN1 cells grown on Cytodex-3 microcarrier beads. Images in parts **a**, **b**, **c** courtesy of NASA, USA.

plant growth in microgravity. These studies revealed that good growth was obtained in reduced gravity, compared with that observed in ground controls, for plants exposed to 12-hour periods of light delivery using red and blue light-emitting diodes[175,176]. This space-based light technology was subsequently shown to have on-Earth applications for enhancing natural healing in a mouse model of diabetes that exhibits

Box 2 | **Summary of cell changes observed in microgravity**

Exposure to microgravity may result in cellular alterations that affect structure and function. In cells grown in space, various changes have been observed, including cytoskeleton rearrangement, coincident with a more rounded cell shape; changes in cell signalling, including alterations to protein kinase C distribution and immune cell cytokine release; and changes in gene expression, ranging from genes regulating cell morphology to those involved in replication, tumour suppression and signal transduction (see the figure).



impaired wound healing[177] and for the treatment of oral mucositis in a study of patients undergoing bone marrow transplant[178]. In this clinical trial, the ability of extra-oral application of near-infrared light at 670 nm (administered daily for 2 weeks) was shown to significantly reduce patient-reported pain in a randomized, double-blind placebo-controlled trial of patients receiving myeloablative treatment followed by haematopoietic stem cell transplant for a variety of malignancies, including leukaemias, lymphomas, myeloma, Ewing's sarcoma and neuroblastoma, in addition to other haematological syndromes (for example, sickle cell anaemia, myelodysplastic syndrome and haemophagocytic lymphohistiocytosis). The investigators postulated that the mechanism of pain relief induced by light therapy may occur via the downregulation of the inflammatory process, including reduced production of the pro-inflammatory cytokine IL-1, as well as diminished production of prostaglandins via inhibition of cyclooxygenase 2 (COX2), as has been suggested in other trials of light therapy[179,180].

Certain light therapy studies carried out to improve astronaut health may also be applicable to increasing our understanding of cancer. Because the spaceflight environment is challenging and characterized by heightened stress, sleeplessness and fatigue, investigations into strategies to counteract these effects have been explored. Exposure to blue light at 420 nm to 460 nm induces potent suppression of melatonin, which can result in increased alertness[181–183]. Originally examined for the mitigation of circadian disruption that is associated with shift work and travel across multiple time zones, this approach may provide a non-pharmacological alternative to facilitate a heightened state of awareness in orbit. However, studies suggest that prolonged suppression of melatonin and disruption of the sleep–wake cycle may be associated with an increased risk for breast cancer[184–187] and colorectal cancer[188]. Taken collectively, data obtained from space-based studies in light therapy offer insight into a range of outcomes that affect cancer and human health.

Conclusions and outlook

Space presents an unlimited horizon for investigation and discovery. Controlled studies conducted in microgravity can further our understanding of the fundamental role of gravity in cancer cell growth and function and serve as a novel paradigm for innovation. Suspension-based 3D cell culture is a useful complement for providing some aspects of growth in microgravity, including the development of spatially unrestricted growth, the generation of multicellular tissue-like architecture, and the support of colocation of cells of different types and size. In order to address the complexities of cancer growth and regulation, a broad view must be adopted that engages all capabilities inherent in model systems that are applicable to the study of this devastating disease. Combination of the resources available in the unique environment of microgravity with the tools and advanced technologies that exist in laboratories across Earth may inform new research approaches to expand the knowledge necessary for improving treatment options, and enhancing the quality of life for those affected by this illness.

Oral mucositis
Inflammation and ulceration occurring in the mouth, often experienced as a side effect of receiving cancer chemotherapy.

Myeloablative treatment
The use of antitumour therapy to eliminate cancer in the bone marrow.

Light therapy
Administration of varying wavelengths of light to affect a biological outcome.

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