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Cytoskeleton changes of mammalian cells in microgravity: results from three decades of low-gravity research

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With the onset of human space flight, the profound consequences of microgravity (or weightlessness) on living organisms became apparent. Subsequently, understanding the biological processes and developing effective countermeasures has moved into the research focus. Despite their small size, isolated cells also show many adaptations in microgravity, but many fundamental processes are not understood. Because the cytoskeleton largely determines cells' mechanical properties and is thought to play an important role in cellular mechanosensing, cytoskeleton adaptation in microgravity have been the focus of many research studies. More than 35 years ago, microtubules assembled in a cell-free system were demonstrated to be gravity dependent. Since then, multiple studies have described cytoskeleton adaptations in varieties of cells exposed to short- or long-term microgravity. In this paper on cytoskeletons in microgravity research, I aimed to grasp the published results as a bigger picture and quantify the reported effects in a systematic and more objective manner. The paper focuses on mammalian cells exposed to real microgravity (free fall) and starts with a brief review on the mechanisms how cells can or could sense their physical environment and the role of the cytoskeleton in mechanobiology.

KEYWORDS

cytoskeleton, actin, tubulin, intermediate filament, microgravity, space flight

1 Introduction

Life has evolved under the constant influence of Earth's gravity, and as a result, all organisms are well adapted to this constant force (Jamon, 2014; Najrana and Sanchez-Esteban, 2016; Vinogradova et al., 2021; Volkmann and Baluska, 2006). Many higher organisms, including plants and vertebrates, have gravity-sensitive structures for orientation. In the early days of space flight, there were major concerns if humans can survive in microgravity (or weightlessness). Pioneering test flights with dogs and monkeys, demonstrated that microgravity is not an acute lethal condition for mammals (Burgess and Dubbs, 2007). With the subsequent evolution of human space flight, the profound influence of microgravity on living organisms became apparent (Blaber et al., 2010; Clément, 2007; Demontis et al., 2017; Williams et al., 2009). Since then, understanding the fundamental biological processes of these adaptations and developing effective countermeasures has moved into the research focus.

Despite their small size, isolated cells in cell cultures also show many adaptations in a microgravity environment (Clément and Slenzka, 2006; Grimm et al., 2011; Lv et al., 2023; Nickerson et al., 2015; Wuest et al., 2018). However, to date, it is not clear how

nonspecialized cells (without specific gravity sensing structures or organelles) can "sense" gravity or what fundamental mechanism triggers the adaptation processes in microgravity (Albrecht-Buehler, 1991; Hader et al., 2017; Ingber, 1999). Because the cytoskeleton largely determines cells' mechanical properties (Fletcher and Mullins, 2010; Rajagopal et al., 2018) and is thought to play an important role in the cell's ability to sense its mechanical environment (Eyckmans et al., 2011; Ingber, 2003a; 2003b; Jansen et al., 2015; Wang, 2017), cytoskeleton adaptation in microgravity have been the focus of many research studies. Indeed, more than 35 years ago (published in 1988), microtubules assembled in a cell-free system were shown to be gravity dependent (Moos et al., 1988). Later analysis showed that microtubule filaments align to gravity once they reach a critical length (Papaseit et al., 2000). This is surprising, as one might think that gravitational forces are too small at a molecular level to have an impact. Since then, multiple studies have described cytoskeleton adaptations in varieties of cells exposed to short- or long-term microgravity (Crawford-Young, 2006; Rudimov and Buravkova, 2016; Vorselen et al., 2014; Wu et al., 2022).

In this paper on cytoskeletons in microgravity research, I would like to take a step back and discuss the results from these studies from a wider perspective. This work focuses on microscopy images from mammalian cells exposed to real microgravity (free fall). Studies using simulated microgravity (Brungs et al., 2016; Herranz et al., 2013), such as clinostats (Briegleb, 1992; Klaus et al., 1998), rotating wall vessels (Ayyaswamy and Mukundakrishnan, 2007), random positioning machines (Wuest et al., 2015) or magnetic levitation (Qian et al., 2013) are not included. Also transcriptomics and proteomics studies are omitted and discussed in dedicated reviews (Abdelfattah et al., 2024; Corydon et al., 2023; Graf et al., 2024; Schulz et al., 2022; Strauch et al., 2019). But first, let's briefly review some mechanisms on how cells can or could sense their physical environment and the role of cytoskeletons in mechanobiology.

1.1 Cytoskeletons and mechanomics

Cells are well known as "chemical machines" that are able to integrate, combine and respond to chemical cues from their environment. However, cells also sense and respond to their mechanical environment in an equally complex manner. Cells not only respond to a wide spectrum of mechanical stimulations, such as stretch, compression, vibrations and deformation, but also sense the physical properties of their environment, such as surface topography and substrate and matrix properties. Because cells incorporate, combine and respond to their complex mechanical environment, the terms "mechanomics" (van Loon, 2007; Wang et al., 2014) and "physicomics" (van Loon, 2009) were postulated. The mechanisms of mechanotransduction, which is the process of translating mechanical forces into a biological response (Cao et al., 2024; Huang et al., 2004; Hughes-Fulford and Boonstra, 2010; Humphrey et al., 2014; Janmey and McCulloch, 2007; Ogneva, 2013; Vogel, 2006; Wang et al., 2014; Wang and Thampatty, 2006), are not fully understood and are subjects of ongoing research. In the next section, the properties of cytoskeletal networks are briefly recapped, followed by a discussion on some of the current concepts related to mechanotransduction.

The cytoskeleton is a dense network of protein filaments that spans the entire cytosol (Zampieri et al., 2014). It consists of three major filament types: the rope-like actin filaments, the rod-like microtubules and the large protein family collectively named intermediate filaments (Schatten, 2015). Actin is the most abundant protein found in eukaryotic cells (Gardel et al., 2008). Its filaments are about 5–9 nm in diameter and are assembled from globular actin (G-actin) into a two-stranded helical polymer (F-actin) (Gardel et al., 2008). These flexible actin filaments interact with a large group of actin-binding proteins that help to organize and reorganize actin network architecture (Pollard, 2016). Broadly speaking, actin network architecture can be divided into gel-like networks, in which the filaments show a more random orientation, and bundles, in which the filaments are aligned in parallel. Cross-linked actin filament bundles are referred to as stress fibers (Tojkander et al., 2012), which can act as tension-bearing cables (Kumar et al., 2006). Myosin motor proteins are able to actively pull on the actin filaments and thereby help to organize and pre-stress the actin network (Vale and Milligan, 2000). In contrast, microtubules are long, hollow cylinders assembled from tubulin dimers. This hollow structure, with an outer diameter of 25 nm and an inner diameter of 17 nm, makes microtubules stiff polymers with a high bending rigidity (Gardel et al., 2008; Hawkins et al., 2010). Unlike actin and intermediate filaments, microtubules typically have a single microtubule organizing center (centrosome), from which the microtubules radiate to the cell periphery (Conduit et al., 2015). Microtubules are known to be highly dynamic, showing cycles of rapid growth and depolymerization, which is referred to as dynamic instability (Holy and Leibler, 1994; Mitchison and Kirschner, 1984). Similar to actin, microtubules interact with a large range of microtubule-associated proteins, of which some can cross-link and organize microtubules into bundles (Bodakuntla et al., 2019; Hawkins et al., 2010). Both actin and microtubules are polar molecules and act as intracellular "highways" along which motor proteins transport cargo, such as vesicles or ribonucleoprotein particles (Appert-Rolland et al., 2015; Mogre et al., 2020). Because microtubules are relatively straight, they are especially suitable for cellular trafficking (Barlan and Gelfand, 2017; Burute and Kapitein, 2019; Fletcher and Mullins, 2010; Gardel et al., 2008; Mogre et al., 2020). Finally, intermediate filaments are a heterogeneous protein family with more than 70 proteins in the human genome (Qin et al., 2010; Szeverenyi et al., 2008). They received their name because their filament size (ca. 10 nm) ranges between those of actin and microtubules (Ishikawa et al., 1968). The molecular building blocks of intermediate filaments are fibrous α-helical proteins that assemble into coiled-coil dimers. These dimers associate laterally and longitudinally to form rope-like filament bundles (Gardel et al., 2008). Unlike actin and microtubules, intermediate filaments are nonpolar (Gardel et al., 2008) and are the most flexible of all filaments (Charrier and Janmey, 2016; Fletcher and Mullins, 2010). In contrast to actin and microtubules, intermediate filaments and their networks can withstand large deformations to several times their original length before breaking (Guzmán et al., 2006; Janmey et al., 1991; Kreplak et al., 2005). However, intermediate filaments can also

cross-link to each other as well as to microtubules and actin filaments (Wiche, 1998).

The cytoskeleton is a major contributor to cells' mechanical properties (Fletcher and Mullins, 2010; Ingber, 2003a; 2003b; Rajagopal et al., 2018). Actin and the more elastic intermediate filaments are thought to mainly determine cell stiffness, whereas the stiff microtubules help cells to resist compressive loads (Ingber, 2008). In in vitro experiments, actin (Janmey et al., 1994; Storm et al., 2005; Xu et al., 2000) and intermediate filament (Janmey et al., 1991; Qin et al., 2010; Schopferer et al., 2009; Storm et al., 2005) networks become increasingly stiff with increasing extension under mechanical load (strain-stiffening behavior). However, apart from the individual components of the cytoskeleton, its architecture is a critical contributor to the cell's mechanical properties (Fletcher and Mullins, 2010). The cytoskeletal network is highly cross-linked and prestressed by motor proteins (Ingber, 2003a; 2003b; Jensen et al., 2015). In contrast to the stress-stiffening behavior of cytoskeletal networks (Gardel et al., 2008), weakly cross-linked actin networks (Gardel et al., 2004; Xu et al., 1998) and pure microtubule networks (Lin et al., 2007) showed stress-softening behavior in in vitro experiments. Similarly, microtubules appear to help homogenize strain distribution in actin in in vitro networks (Gardel et al., 2008). This exemplifies the importance of cross-linking proteins in helping the cytoskeletal network resist large stresses and deformations without breaking (Gardel et al., 2008). Furthermore, cells prestress their cytoskeletal network using motor proteins, which is an important contributor to cells' mechanical properties and morphology (Ingber, 2003a; 2003b; Jensen et al., 2015). Therefore, the tubulin network typically appears highly bent in cells (Gardel et al., 2008; Ingber, 2008). This prestressed interplay of flexible filaments (actin and intermediate filaments) with stiff microtubules eventually led to the formulation of the tensegrity model (Ingber, 1993; 2003a, 2003b). Apart from providing mechanical stability to the cells and allowing them to maintain their shape, the cytoskeleton also seems to stabilize intracellular components (Guo et al., 2013). Finally, the cytoskeleton is connected via specific junctions to the extracellular matrix (ECM) and other cells. Focal adhesions (FAs) (Burridge, 2017; Geiger et al., 2009; Wehrle-Haller, 2012) connect the actin cytoskeleton to the ECM, while hemidesmosomes (Borradori and Sonnenberg, 1999; Green and Jones, 1996; Walko et al., 2015) connect intermediate filaments to the cell substrate. Desmosomes (Desai et al., 2009; Green and Jones, 1996; Holthöfer et al., 2007) and adherens junctions (Dejana and Orsenigo, 2013; Niessen and Gottardi, 2008) connect the intermediate and actin networks of neighboring cells, respectively.

To date, several possible mechanisms have been identified concerning how cells might sense their physical environment, including mechanical load (mechanotransduction). These pathways are not mutually exclusive and are likely overlapping, creating an actual mechanosensitive signaling network (Ingber, 1999; Wang et al., 2014). The first instance of mechanosensation is probably not within the cells but in the surrounding ECM. The ECM can act as a reservoir for many signaling molecules, which are released and presented to cells under mechanical load (Wang and Thampatty, 2006). For example, the transforming growth factor-β (TGF-β) can be activated and released from the ECM by contractile forces (Wipff et al., 2007). Furthermore, multiple proteins are known to unravel under mechanical load, exposing cryptic sites that are buried in a native unloaded state (Vogel, 2006). A prominent example is the ECM protein fibronectin, which has many recognition sites binding to other ECM proteins, serum proteins and cell adhesion proteins (Vogel, 2006; Vogel and Sheetz, 2009). Cells are attached via FAs to the ECM, and eventually, physical forces are passed through these protein complexes to the cells (Burridge, 2017; Eyckmans et al., 2011; Geiger et al., 2009; Hughes-Fulford and Boonstra, 2010; Wehrle-Haller, 2012). Members from the integrin protein family are a prominent example among the many proteins that cluster in FAs. Integrins are transmembrane glycoproteins that connect the actin cytoskeleton to ECM proteins (e.g., fibronectin and vitronectin) (Burridge, 2017; Geiger et al., 2009; Huang et al., 2004; Hughes-Fulford and Boonstra, 2010; Ogneva, 2013; Wehrle-Haller, 2012). Integrin binding to the ECM can activate transcription factors via the focal adhesion kinase (FAK), activated intermediate messengers and the MAP kinase (Hughes-Fulford and Boonstra, 2010). Not surprisingly, integrin is known to regulate various cellular functions, including cell attachment, proliferation, migration and differentiation (Coppolino and Dedhar, 2000). The formation and maturation of FAs requires the application of force (Eyckmans et al., 2011; Geiger and Bershadsky, 2001; Geiger et al., 2009; Hughes-Fulford and Boonstra, 2010). Correspondingly, inhibition of contractile forces leads to a disassembly of FAs (Chrzanowska-Wodnicka and Burridge, 1996; Hughes-Fulford and Boonstra, 2010). In a similar matter, cytoskeleton stability is likely dependent on acting forces. For instance, the pitch length of helical actin filaments increases under tensile force. This increases actin's affinity to myosin II and reduces the affinity to cofilin, which is known to sever filamentous actin (Hayakawa et al., 2011; McGough et al., 1997).

Inside the nucleus, a dense network of lamins (members of intermediate filaments) and nuclear lamin-associated membrane proteins form the nuclear lamina (Gruenbaum and Foisner, 2015; Wang et al., 2009). The nuclear lamina is crucial for nuclear organization and involved in several cellular functions, including activation of transcription factors (Dorner et al., 2006; Ho et al., 2013; Ivorra et al., 2006; Osmanagic-Myers et al., 2015), regulation of chromatin epigenetic state (Harr et al., 2015; Solovei et al., 2013), chromosome tethering (Gruenbaum and Foisner, 2015) and cell polarization and migration (Davidson et al., 2014; Harada et al., 2014; Lee et al., 2007). The lamina is connected to the cytoskeletal network via the LINC complex (linker of nucleoskeleton and cytoskeleton) (Crisp et al., 2005; Sosa et al., 2013). It is therefore thought that forces transmitted via the cytoskeleton to the nucleus can lead to nuclear deformation, which modulates transcription and chromatin organization (Kirby and Lammerding, 2018; Uhler and Shivashankar, 2017; Wang et al., 2009).

Finally, the cell membrane and its associated proteins (Anishkin and Kung, 2013; Ingber, 2006), particularly the mechanosensitive ion channels (MSCs), have been proposed as mechanosensors as well. MSCs, also known as stretch-activated channels, are characterized by the ability to switch between closed and open states in response to mechanical load (Lecar and Morris, 1993; Sachs, 2010; Sachs and Morris, 1998). This conformational change allows specific ions (e.g., sodium, potassium and calcium) to cross the plasma membrane under local mechanical membrane tension (Wang and Thampatty, 2006). While it is not fully clear how these

TABLE 1 Reported effects of actin adaptations in microgravity sorted from strong effect to no observable changes. Every line represents a reported claim in a publication. Therefore, publications with multiple claims and timepoints appear multiple times.

Score	Organism	Cell	Cell type	Platform	Duration	Microgravity effect compared to Earth gravity	Reference
Fair to strong	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	3 days	Decreased amount of F-actin	Li et al. (2018)
Fair to strong	Wistar rats	Osteosarcoma ROS 17/2.8	Cell line	Foton	4 days	Disappearance of large bundles of stress fibers	Guignandon et al. (2001)
Fair to strong	Human	Follicular thyroid carcinoma cells (FTC-133)	Cell line	Sounding rocket	6 min	Appearance of filopodia-, and lamellipodia-like structures	Corydon et al. (2016)
Fair to strong	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	3 days	F-actin accumulation at the cellular membrane	Li et al. (2018)
Fair to strong	Mouse	Osteoblast (MC3T3-E1)	Cell line	Space shuttle	4 days	Reduced stress fibers	Hughes-Fulford and Lewis (1996)
Fair	Human	Monocytes	Cell line	ISS	1 day	Decreased density of the filamentous biopolymers of F-actin	Meloni et al. (2011)
Fair	Human	U937 differentiated to macrophageal	Cell line	Shenzhou	5 days	Loss of actin fluorescence staining	Paulsen et al. (2014)
Fair	Human	Monocytes	Cell line	ISS	1 day	Disappearance of the complex actin cytosolic network	Meloni et al. (2011)
Fair	Human	Primary macrophages	Primary	ISS	30 days	Reduced F-actin fluorescent intensities	Tauber et al. (2017)
Fair	*Xenopus*	Myocytes	Primary	Space shuttle	9 days	Predominant distribution of actin filaments showed thick ("ropy") filaments	Gruener et al. (1994)
Fair	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	10 days	Decreased amount of F-actin	Li et al. (2018)
Fair	Human	Monocytes	Cell line	ISS	1 day	Actin filaments appeared mostly localized, close to the plasma membrane	Meloni et al. (2011)
Fair	Mouse	Primary osteoblast	Primary	Foton	5 days	Thinner cortical actin	Nabavi et al. (2011)
Small to fair	Wistar rat	Neonatal cardiac myocytes	Primary	Parabolic flight	3 h	Actin filaments were more prominent and thick	Yang et al. (2010)
Small to fair	Human	MCF-7 breast cancer cells	Cell line	Sounding rocket	2.5 min	Nonspecific changes in the F-actin network	Nassef et al. (2019)
Small to fair	Human	Follicular thyroid carcinoma cells (FTC-133)	Cell line	Sounding rocket	6 min	Disturbance of F-actin bundles	Corydon et al. (2016)
Small to fair	Wistar rats	Osteosarcoma ROS 17/2.8	Cell line	Foton	12 h	Decreased number of actin stress fibers	Guignandon et al. (2003a)
Small to fair	Mouse	Primary osteoblast	Primary	Foton	5 days	Thinner actin stress fibers	Nabavi et al. (2011)
Small to fair	Human	Follicular thyroid carcinoma cells (FTC-133)	Cell line	Parabolic flight	20 s	Disappearance of microvilli or filopodia- and lamellipodia-like structures	Corydon et al. (2016)
Small	Human	MCF-7 breast cancer cells	Cell line	Sounding rocket	2.5 min	Local F-actin accumulations	Nassef et al. (2019)
Small	Wistar rat	Neonatal cardiac myocytes	Primary	Parabolic flight	3 h	Increased F-actin (polymer microfilament) and decreased G-actin (free monomer), which resulted in a shift to F-actin in the F/G-actin equilibrium	Yang et al. (2010)
Small	Human	Chondrocytes	Primary	Parabolic flight	1 min	Actin ring formed around the cell membrane	Aleshcheva et al. (2015)
Small	Human	Chondrocytes	Primary	Parabolic flight	2 h	Actin ring formed around the cell membrane	Aleshcheva et al. (2015)

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TABLE 1 (*Continued*) Reported effects of actin adaptations in microgravity sorted from strong effect to no observable changes. Every line represents a reported claim in a publication. Therefore, publications with multiple claims and timepoints appear multiple times.

Score	Organism	Cell	Cell type	Platform	Duration	Microgravity effect compared to Earth gravity	Reference
Small	Wistar rats	Osteosarcoma ROS 17/2.8	Cell line	Foton	4 days	Cortical pattern of actin network	Guignandon et al. (2001)
No to small	*Xenopus*	Myocytes	Primary	Space shuttle	9 days	Predominant distribution of actin filaments, which were frequently not aligned with the long axis of the cell and had a nonlinear ("wavy") appearance	Gruener et al. (1994)
No to small	Mouse	Osteoblast (MC3T3-E1)	Cell line	Space shuttle	4 days	Elongated extended filopodia	Hughes-Fulford and Lewis (1996)
No to small	Human	Neuroblastoma (SH-SY5Y)	Cell line	Parabolic flight	18 min	Enhanced actin-driven protrusion of evoked lamellipodia	Rösner et al. (2006)
No to small	Human	Epidermoid carcinoma cells (A431)	Cell line	Sounding rocket	6 min	Enhanced relative filamentous actin content	Rijken et al. (1992)
No to small	Human	Follicular thyroid carcinoma cells (FTC-133)	Cell line	Parabolic flight	20 s	"Holes" in the actin network	Corydon et al. (2016)
No to small	Human	Follicular thyroid carcinoma cells (FTC-133)	Cell line	Parabolic flight	20 s	Disturbance of F-actin bundles	Corydon et al. (2016)
No to small	Human	Primary macrophages	Primary	ISS	30 days	Fewer cells showed "strings"	Tauber et al. (2017)
No to small	Human	Thyroid cancer cells (ML-1)	Cell line	Parabolic flight	22 s	Rearrangement of the actin network with perinuclear clustering	Ulbrich et al. (2011)
No	Human	T lymphoblastoid (Jurkat)	Cell line	Sounding rocket	12 min	No effect reported	Cogoli-Greuter et al. (1997)
No	Pic	Chondrocytes	Primary	Sounding rocket	13 min	No effect reported	Conza et al. (2003)
No	Human	Fibroblast	Primary	Sounding rocket	6 min	No effect reported	Jongkind et al. (1996)
No	Human	MCF-7 breast cancer cells	Cell line	Sounding rocket	2.5 min	Appearance of filopodia- and lamellipodia-like structure	Nassef et al. (2019)
No	Human	T lymphoblastoid (Jurkat)	Cell line	Sounding rocket	12 min	No effect reported	Sciola et al. (1999)
No	Human	Primary macrophages	Primary	ISS	11 days	No effect reported	Tauber et al. (2017)
No	Wistar rat	Neonatal cardiac myocytes	Primary	Shenzhou	4 days	No effect reported	Yang et al. (2008)
No	Wistar rat	Neonatal cardiac myocytes	Primary	Shenzhou	48 days	No effect reported	Yang et al. (2008)
No	Wistar rat	Neonatal cardiac myocytes	Primary	Shenzhou	96 days	No effect reported	Yang et al. (2008)

channels are coupled to mechanical forces, it is thought that some MSCs are linked directly or indirectly to the cytoskeleton or the ECM, and others interact only with the surrounding lipids (Anishkin et al., 2014; Hamill, 2006; Hayakawa et al., 2008; Sachs, 2010; Sachs and Morris, 1998). All membrane proteins, including MSCs, must match the hydrophobicity of the lipid bilayer. As mechanically stressed membranes become thinner under tension (Reddy et al., 2012), this could lead to hydrophobic mismatches at the protein–lipid interface, which could favor MSCs to undergo a conformational change (Anishkin et al., 2014).

In summary, the cytoskeleton plays a central role in numerous cellular functions. It largely determines cells' mechanical stability and morphology. The cytoskeleton is also critically important for intracellular organization and molecular transport. It acts as a scaffold for many cellular organelles (Charrier and Janmey, 2016; Guo et al., 2013; Hughes-Fulford and Boonstra, 2010), and motor proteins transport their cargo along microtubules and actin filaments (Appert-Rolland et al., 2015; Mogre et al., 2020). Cells can selectively prestress or relax the cytoskeletal network via motor proteins and cytoskeleton remodeling (Ingber, 2003a; Jansen et al., 2015; Wang, 2017). Constant remodeling also allows cells to enforce

TABLE 2 Reported effects of microtubule adaptations in microgravity sorted from strong effect to no observable changes. Every line represents a reported claim in a publication. Therefore, publications with multiple claims and timepoints appear multiple times.

Score	Organism	Cell	Cell type	Platform	Duration	Microgravity effect compared to Earth gravity	Reference
Fair to strong	Wistar rat	Neonatal cardiac myocytes	Primary	Parabolic flight	3 h	Disrupted microtubules	Yang et al. (2010)
Fair to strong	Human	Neuroblastoma (SH-SY5Y)	Cell line	Parabolic flight	18 min	Altered arrangement of microtubules indicated by bending, turning and loop formation	Rösner et al. (2006)
Fair to strong	Wistar rat	Neonatal cardiac myocytes	Primary	Parabolic flight	3 h	Straight microtubular filaments on the periphery were intermittent (interrupted)	Yang et al. (2010)
Fair to strong	Human	Promyelocytic leukocyte cell line (HL60)	Cell line	Space shuttle	3 days	Lack of cytoskeletal polymerization, resulting in an overall amorphic globular shape	Piepmeier et al. (1997)
Fair to strong	Human	Breast cancer cells (MCF-7)	Cell line	Foton	48 h	Microtubules appeared more diffuse	Vassy et al. (2001)
Fair to strong	Wistar rat	Neonatal cardiac myocytes	Primary	Parabolic flight	3 h	Decreased microtubules in perinuclear regions	Yang et al. (2010)
Fair	Human	U937 differentiated to macrophages	Cell line	Shenzhou	5 days	Tubulin accumulated and clumped; the whole cell, including the nuclear region, was filled with tubulin fibers, with the exception only of vacuole-like regions	Paulsen et al. (2014)
Fair	Sea urchin	Embryos	Primary	Space shuttle	3 h	4% of all cells undergoing division showed abnormalities in the centrosome-centriole complex, while most cells showed a normal mitotic apparatus	Schatten et al. (1999)
Fair	Human	MCF-7 breast cancer cells	Cell line	Sounding rocket	2.5 min	Accumulations, holes and looser structures	Nassef et al. (2019)
Small to fair	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	3 days	Decreased amount of microtubules	Li et al. (2018)
Small to fair	Human	Endothelial EA.hy926 cells	Cell line	Parabolic flight	20 s	Rearrangement of tubulin with accumulations around the nucleus	Grosse et al. (2012)
Small to fair	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	10 days	Decreased amount of microtubules	Li et al. (2018)
Small to fair	Human	U937 differentiated to macrophages	Cell line	Shenzhou	5 days	Increased fluorescent signal of tubulin staining	Paulsen et al. (2014)
Small to fair	Wistar rat	Neonatal cardiac myocytes	Primary	Shenzhou	48 h	Diffuse tubulin staining and some dotted spot staining indicated the fragmentation of microtubular filaments; microtubules at periphery were intact	Yang et al. (2008)
Small to fair	Human	Monocytes	Primary	ISS	1 day	Disrupted tubulin architecture	Meloni et al. (2011)
Small to fair	Human	U937 differentiated to macrophages	Cell line	Shenzhou	5 days	Disorganized tubulin skeleton	Paulsen et al. (2014)
Small to fair	Human	T lymphoblastoid (Jurkat)	Cell line	Space shuttle	4 h	Tubulin filaments were shortened and coalesced, and they lacked normal branching at the cell membrane	Lewis et al. (1998)
Small to fair	Human	T lymphoblastoid (Jurkat)	Cell line	Space shuttle	4 h	Disrupted microtubule organizing centers (MTOCs)	Lewis et al. (1998)
Small to fair	Wistar rat	Neonatal cardiac myocytes	Primary	Shenzhou	96 h	Depolymerization of tubulin	Yang et al. (2008)
Small	Rat	Primary vestibular hair cells	Primary	Foton	5 days	Tubulin was organized into filaments, but had no bundles	Gaboyard et al. (2002)
Small	Mouse	Primary osteoblast	Primary	Foton	5 days	Shorter, thicker and wavier (looped) microtubules	Nabavi et al. (2011)

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TABLE 2 (*Continued*) Reported effects of microtubule adaptations in microgravity sorted from strong effect to no observable changes. Every line represents a reported claim in a publication. Therefore, publications with multiple claims and timepoints appear multiple times.

Score	Organism	Cell	Cell type	Platform	Duration	Microgravity effect compared to Earth gravity	Reference
Small	Human	Breast cancer cells (MCF-7)	Cell line	Foton	1.5 h	Appearance of tubulin labeled lamellipodia	Vassy et al. (2001)
Small	Human	Breast cancer cells (MCF-7)	Cell line	Foton	48 h	Shorter microtubules	Vassy et al. (2001)
Small	Human	Breast cancer cells (MCF-7)	Cell line	Foton	1.5 h	Instead of long, strongly labeled microtubules radiating throughout the cytoplasm, only a few filaments could be distinguished against the strong (gray) background	Vassy et al. (2001)
Small	Human	Breast cancer cells (MCF-7)	Cell line	Foton	22 h	The cytoplasm was less clear	Vassy et al. (2001)
Small	Wistar rat	Neonatal cardiac myocytes	Primary	Parabolic flight	3 h	Obtuse protrusive edges with looped microtubules	Yang et al. (2010)
No to small	Rat	Primary vestibular hair cells	Primary	Foton	5 days	Defasciculated tubulin filaments; tubulin was not specifically located in the neck of the cells	Gaboyard et al. (2002)
No to small	Mouse	Primary osteoblast	Primary	Foton	5 days	Shorter acetylated microtubules with more curls at more acute angles	Nabavi et al. (2011)
No to small	Human	Chondrocytes	Primary	Parabolic flight	2 h	Formation of holes	Aleshcheva et al. (2015)
No to small	Human	Breast cancer cells (MCF-7)	Cell line	Foton	22 h	Shorter microtubules	Vassy et al. (2001)
No to small	Wistar rat	Neonatal cardiac myocytes	Primary	Shenzhou	4 h	Diffuse tubulin staining and some dotted spot staining indicated the fragmentation of microtubular filaments; microtubules at periphery were intact	Yang et al. (2008)
No to small	Human	Chondrocytes	Primary	Parabolic flight	2 h	Depolymerization of the tubulin network	Aleshcheva et al. (2015)
No	Human	Chondrocytes	Primary	Parabolic flight	1 min	No effect reported	Aleshcheva et al. (2015)
No	Pic	Chondrocytes	Primary	Sounding rocket	13 min	No effect reported	Conza et al. (2003)
No	Wistar rats	Osteosarcoma ROS 17/2.8	Cell line	Foton	12 h	No effect reported	Guignandon et al. (2003b)
No	Human	T lymphoblastoid (Jurkat)	Cell line	Space shuttle	48 h	No effect reported	Lewis et al. (1998)
No	Bovine	Chondrocytes	Primary	Parabolic flight	2 h	No effect reported	Wuest et al. (2020)

or weaken the cytoskeleton to adapt to the governing forces (Kobayashi and Sokabe, 2010; Ohashi et al., 2017; Tojkander et al., 2012; Wang, 2017). In cellular mechanosensation, the cytoskeleton can dissipate mechanical forces (to reduce machanosensitivity) and channel mechanical forces to mechanosensitive structures, such as FAs, the nucleus or MSCs. These mechanical signals propagate through the prestressed cytoplasm much more quickly than diffusion-based chemical signals (Jaalouk and Lammerding, 2009).

2 Results

Due to the cytoskeleton's central role in cellular mechanics and mechanosensation, the cytoskeleton was quickly thought to be gravity dependent. During the past three decades, numerous researchers have used isolated cell cultures to investigate cytoskeleton adaptations in (real) microgravity (Lewis, 2004; Rudimov and Buravkova, 2016; Vorselen et al., 2014; Wu et al., 2022), making use of parabolic flights (Karmali and Shelhamer, 2008; Pletser, 2016; Pletser et al., 2016), sounding rockets (Seibert, 2006) and orbiting spacecrafts (Duan and Long, 2019; NASA, 2010; Rai et al., 2016). The published data describe a large variety of cytoskeleton adaptations already within a minute after exposure to microgravity. Unfortunately, there is no clear pattern concerning how microgravity affects the cytoskeleton. Confounding factors could be the variety in study designs, types of hardware, cell sources and constraints of microgravity platforms. A minority of the authors reported not having detected any changes (negative finding; refer to Tables 1–3).

TABLE 3 Reported effects of intermediate filament adaptations in microgravity sorted from strong effect to no observable changes. Every line represents a reported claim in a publication. Therefore, publications with multiple claims and timepoints appear multiple times.

Score	Filament	Organism	Cell	Cell type	Platform	Duration	Microgravity effect compared to Earth gravity	Reference
Fair to strong	Vimentin	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	10 days	Increased vimentin expression	Li et al. (2018)
Fair to strong	Vimentin	Human	Macrophages	Primary	ISS	30 days	Reduced vimentin fluorescent intensities	Tauber et al. (2017)
Fair to strong	Vimentin	Human	Chondrocytes	Primary	Parabolic flight	2 h	Formation of holes	Aleshcheva et al. (2015)
Fair to strong	Cytokeratin	Human	Chondrocytes	Primary	Parabolic flight	2 h	Formation of holes	Aleshcheva et al. (2015)
Fair to strong	Vimentin	Human	Macrophages	Primary	ISS	30 days	Fewer cells showed vimentin strings	Tauber et al. (2017)
Fair to strong	Cytokeratin	Human	Breast cancer cells (MCF-7)	Cell line	Foton	48 h	Some unusual patterns	Vassy et al. (2003)
Fair	Vimentin	Human	Macrophages	Primary	ISS	30 days	More cells showed clusters	Tauber et al. (2017)
Small to fair	Vimentin	Human	Macrophages	Primary	ISS	30 days	Fewer cells showed clouds	Tauber et al. (2017)
Small to fair	Cytokeratin	Human	Breast cancer cells (MCF-7)	Cell line	Foton	22 h	The meshes of the network were often looser	Vassy et al. (2003)
Small to fair	Vimentin	Human	T lymphoblastoid (Jurkat)	Cell line	Sounding rocket	12 min	Changes in the structure	Cogoli-Greuter et al. (1997)
Small to fair	Vimentin	Human	T lymphoblastoid (Jurkat)	Cell line	Sounding rocket	12 min	Appearance of large bundles	Cogoli-Greuter et al. (1997)
Small to fair	Cytokeratin	Human	Thyroid cancer cells (ML-1)	Cell line	Parabolic flight	22 s	Clustering of cytokeratin	Ulbrich et al. (2011)
Small	Cytokeratin	Human	Breast cancer cells (MCF-7)	Cell line	Foton	48 h	The meshes of the network were often looser	Vassy et al. (2003)
No to small	Cytokeratin	Human	Breast cancer cells (MCF-7)	Cell line	Foton	1.5 h	The meshes of the network were often looser	Vassy et al. (2003)
No	Vimentin	Human	Chondrocytes	Primary	Parabolic flight	1 min	No effect reported	Aleshcheva et al. (2015)
No	Vimentin	Pic	Chondrocytes	Primary	Sounding rocket	13 min	No effect reported	Conza et al. (2003)
No	Vimentin	Mouse	Osteoblast (MC3T3-E1)	Cell line	Space shuttle	4 days	No effect reported	Hughes-Fulford and Lewis (1996)
No	Vimentin	Human	Endothelial cells (EA.hy926)	Cell line	Satellite	3 days	No effect reported	Li et al. (2018)
No	Vimentin	Human	U937 differentiated to macrophages	Cell line	Shenzhou	5 days	No effect reported	Paulsen et al. (2014)
No	Vimentin	Human	Macrophages	Primary	ISS	11 days	No effect reported	Tauber et al. (2017)
No	Vimentin	Bovine	Chondrocytes	Primary	Parabolic flight	2 h	No effect reported	Wuest et al. (2020)
No	Cytokeratin	Human	Chondrocytes	Primary	Parabolic flight	1 min	No effect reported	Aleshcheva et al. (2015)

However, the extent to which published data is biased toward positive findings is unclear.

To get a more objective view, I have compiled the results of 30 publications and asked ten individuals who were familiar with cytoskeleton staining to score the authors' claims. The allowed scores were "no" change, "small" (barely visible), "fair" (clearly visible) or "strong" (obvious) change. All information regarding the authors, research institutions, utilized microgravity platform or cell source was removed to avoid biasing the scoring. Additionally, the participating individuals were not familiar with the publications.

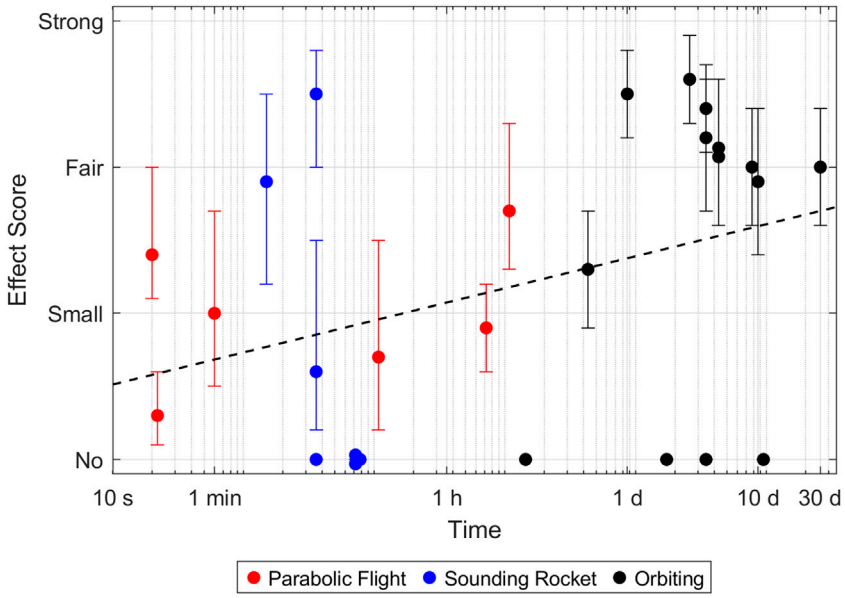

FIGURE 1
Effect size of actin adaptations in microgravity. Every point represents a reported timepoint of a publication. If the authors stated multiple claims, every claim was scored separately and the highest score of each claim was taken. The score represents the average over all participants. Error bars indicate the 95% confidence interval. A complete parabola during a parabolic flight lasts around 1 minute. Samples which were fixed later, therefore went through multiple parabolas and experienced multiple hyper- and microgravity phases.

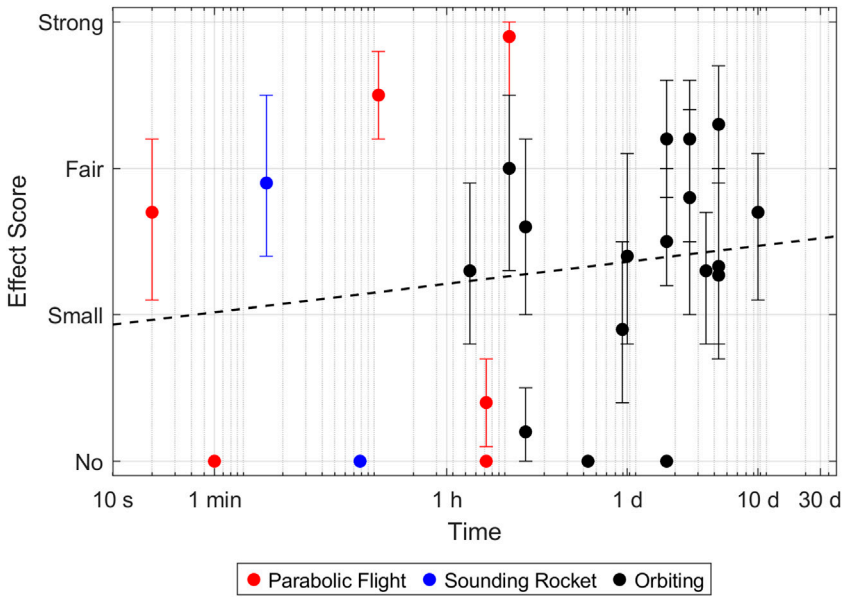

FIGURE 2
Effect size of microtubule adaptations in microgravity. Every point represents a reported timepoint of a publication. If the authors stated multiple claims, every claim was scored separately and the highest score of each claim was taken. The score represents the average over all participants. Error bars indicate the 95% confidence interval. A complete parabola during a parabolic flight lasts around 1 minute. Samples which were fixed later, therefore went through multiple parabolas and experienced multiple hyper- and microgravity phases.

Publications in which researchers investigated multiple proteins (e.g., actin and tubulin) or time points were split, and the proteins and timepoints were scored separately. Publications that reported no change were not displayed for scoring and directly scored as showing "no" change. Figures 1–3 highlight the diversity of the data set (Please refer to the Supplementary Material for a more

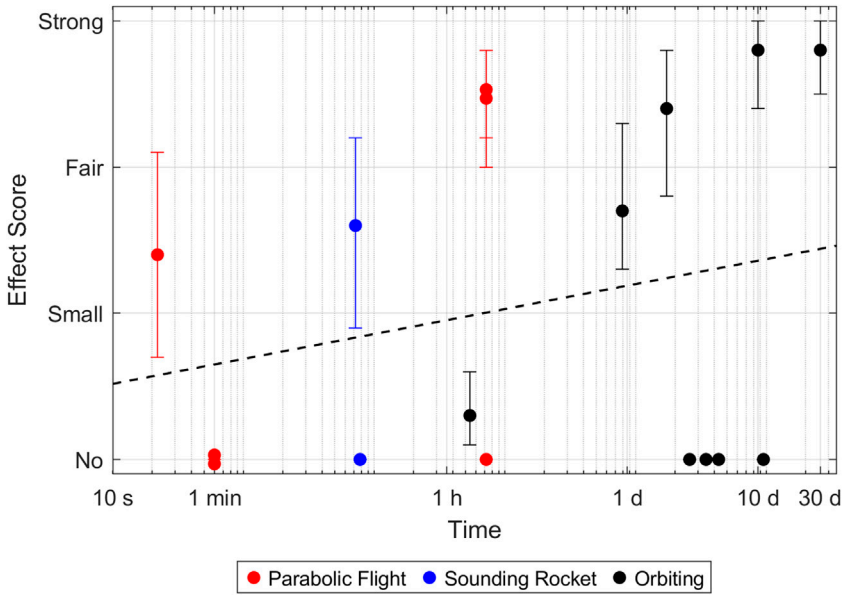

FIGURE 3
Effect size of intermediate filament adaptations in microgravity. Every point represents a reported timepoint of a publication. If the authors stated multiple claims, every claim was scored separately and the highest score of each claim was taken. The score represents the average over all participants. Error bars indicate the 95% confidence interval. A complete parabola during a parabolic flight lasts around 1 minute. Samples which were fixed later, therefore went through multiple parabolas and experienced multiple hyper- and microgravity phases.

detailed description of the data). Generally speaking, orbiting platforms tend to trigger larger cytoskeleton adaptations in microgravity, even though statistically not significant (Wilcoxon rank-sum test with an α of 5%, Supplementary Figure S1). However, time in microgravity seems to be the most dominant factor. (For publications that did not report a timeline, it was estimated from what could be expected from the respective microgravity platform). There is no clear detectable pattern of effect scores with regards to cell culture, cell types (primary or cell line) or cell sources.

The time-dependent effect is most striking for the actin network (The Pearson correlation coefficient of the log-transformed time versus the effect score was computed to be statistically significant with a p-value of 3.2%, Supplementary Table S1). Most experiments that reported "fair" to "strong" effects were done on orbiting platforms. They most often described a decrease or loss of F-actin (Table 1). Similarly, a reduction or loss of stress fibers was reported several times. A few papers reported rearrangements of F-actin such as atypical accumulation, appearance of filopodia- and lamellipodia-like structures (Corydon et al., 2016) or formation of actin filaments. However, effects that could be summarized as actin rearrangement were generally considered small and did not score higher than "fair".

For the tubulin network, the results appear to be much more random and rather independent of time and employed microgravity platform. (Neither the Pearson correlation coefficient nor the Wilcoxon rang-sum test computed a statistically significant differences with p-values lower than 5%, Supplementary Table S1; Supplementary Figure S1). The most often reported effect (also in the "fair" to "strong" range) was a disrupted and more diffuse tubulin network (Table 2). Many papers also reported altered arrangements such as accumulations and holes. In a few experiments, shorter microtubules or filaments were observed, but overall, this seems to have been a rather minor effect.

Finally, a few experiments on the two intermediate filaments, vimentin and cytokeratin, were published. Overall, the effect of microgravity on intermediate filaments also seems to be time dependent at first. However, this claim could not be supported by Pearson correlation statistics (Supplementary Table S1) and the number of publications with positive and negative findings are more or less balanced for all platforms. Also, the findings are rather conflicting. For example, a reduction of vimentin was only reported in one experiment on human macrophages after 30 days in microgravity (Tauber et al., 2017). In contrast, an experiment with human endothelial cells reported an increase of vimentin expression after 10 days in microgravity (Li et al., 2018). Overall, most papers reported some sort of rearrangement, such as formation of holes, clusters, looser structures or formation of bundles (Table 3).

3 Discussion and conclusion

This work aimed at assessing whether the reported changes in the cytoskeleton in microgravity were substantial or relatively minor. The subjective scores of ten blinded individuals, confirmed the heterogeneous nature of previously published findings. Microgravity experiments are challenging in many aspects (Beysens and van Loon, 2015). All microgravity platforms, being parabolic flights, sounding rockets and orbiting space crafts, have a hypergravity period before the actual microgravity phase, which could influence the cellular response. (Due to the short microgravity period, none of the publications

analyzed in this paper made use of a drop tower (Dittus, 1991).) For parabolic flights (typically around 1.5–2 g (Karmali and Shelhamer, 2008)) and large space crafts (typically around 3–5 g), the hypergravity level is rather modest. For sounding rockets, the launch conditions, typically lasting around 45 s, can be rather harsh, with peak linear accelerations of around 13 g accompanied by strong vibrations (Seibert, 2006). Furthermore, all vehicles show an inherent distinct pattern of rising and falling acceleration loads with different timings. For examples, the parabola of a parabolic flight typically last around 1 minute with two hypergravity periods of around 20 s before and after the microgravity phase (Karmali and Shelhamer, 2008). During one flight, often many parabolas are flown every few minutes. In contrast, a two-stage sounding rocket typically experiences about 8 g during the burn of the first stage, then a short microgravity phase (ca. 5 s) during stage separation and peak accelerations up to 13 g during the second stage burn. All microgravity platforms also have specific limitations in accessibility, available experiment space, timelines, environment stability and safety constraints. For orbiting space crafts, the up- and download conditions can be rather problematic. Launch scraps, operational constraints, reentry heat and long recoveries can interfere with the experiment timeline and environment conditions (e.g., optimal temperature range). The high costs related to space-flown experiments and the limited access to microgravity, lead to the fact that all experiments are unique "single-shot" experiments with different cell sources, platforms, hardware, timelines, microgravity qualities, environment conditions and data acquisition protocols. Finally, the 1 g control samples were often acquired under different conditions as well. While some experiments use an in-flight 1 g control, other studies used lab samples or hardware control samples, which were produced either in parallel or after the flight. All of these confounding factors can make data interpretation difficult. This is also mirrored by the very heterogeneous findings reported over the last decades. However, a real and global effect, which is present in many cell types, should still consistently appear in several studies.

This paper quantified whether the reported effects were clear and reasonable or rather small. One must keep in mind that researchers are inclined to look for positive findings and therefore tend to overrate their findings. Negative findings are also much more at risk of not being published (Baker, 2016; Editorial, 2019). The individuals who did the scoring were only shown the claims the authors published and were unaware of the study design, microgravity platform or timeline to prevent potential bias by this information. This resulted in a more generalized, quantified and objective picture than a list of qualitative findings. However, one limitation we faced was that the quality and presentation of the data were also very heterogeneous. The technological and methodological advances between the first experiments and the modern day were also striking. Furthermore, interpretation of cytoskeleton staining remains a qualitative task which is subjected to individual views, backgrounds and interpretations. Therefore, the raters showed strong agreement for some claims but also very diverse answers for other claims (Supplementary Tables S8, S12, S16). This is also reflected in the inter-rater reliability, which was computed to show a "slight agreement" by Fleiss's κ (Cardillo, 2007). Finally, while great effort was invested in a thorough literature search, missed publications could bias the results.

For actin and tubulin cytoskeletons, the published data set is large enough to attempt a first quantitative summary. For the intermediate filaments, namely vimentin and cytokeratin, the number of publications is still rather small. Interestingly, the largest effects on actin were clearly observed on orbiting platforms. There is also a clear time dependency, with strong effects mainly reported after an experiment duration of around 1 day. Among them, the most frequent descriptions could be summarized as a decrease or loss of F-actin or F-actin structures (e.g., stress fibers). In contrast, the results for microtubules are rather random and independent of time and microgravity platform. Additionally, for this molecule, the most often reported findings were disruption and loss of microtubulin organization. Concerning the intermediate filaments, the small data set and incoherent reports do not really allow for a solid conclusion. However, network reorganization, rather than loss or disruption, seems to be the dominant effect.

Overall, this suggests that generally, the cytoskeleton is rather stable under short-term microgravity (on the order of minutes), as mostly small adaptations were reported. Large (obvious) changes were mostly reported after the cells had spent multiple hours or days in microgravity. This suggests that microgravity does not trigger an immediate and profound adaptation of the cytoskeleton. It is more likely that cells have trouble maintaining (or remodeling) the cytoskeleton while in microgravity.

The cytoskeleton could play an important role in acute cellular gravity sensing due to its central role in many cellular processes, including mechanotransduction. This view is also supported by the tensegrity model, which stresses the importance that the cytoskeleton needs to be in equilibrium to the governing forces (Wu et al., 2022). However, it is probably unlikely that exposure to microgravity directly and actively induces reorganization or disassembly of the cytoskeleton. It is more likely that maintenance of the cytoskeleton as part of the regular cell cycle is disturbed, particularly for the actin network. As a result, this could lead to altered cytoskeleton architecture, pre-stress and stability (Figure 4). Over a longer period, this may have serious consequences for cells or organisms, as the cytoskeleton is involved in many important cellular processes, such as cell migration and the cell cycle (Figure 4). Potentially the consequences could imply alterations in cellular organization, cell stiffness (tension & compression), attachment to the ECM, as well as dissipation and transmission of forces through the cells. As the cytoskeleton acts also as a scaffold, molecular transport and stability of intracellular organelles could be affected as well. Ultimately, the nucleus is also closely connected to the cytoskeleton, which could affect transcription, chromatin epigenetic, chromosome tethering and cell polarization. This could have potential implication for astronaut health, pathogen vitality and mutations, plant growth in space (space farming), and life science applications in microgravity, such as tissue engineering.

At this point, the underlying mechanisms leading to a disrupted cytoskeleton in microgravity remain speculative. It could be hypothesized that microgravity makes actin filaments more likely to be severed by cofilin (Hayakawa et al., 2011; McGough et al., 1997). However, it could also suggest that in the normal cell cycle,

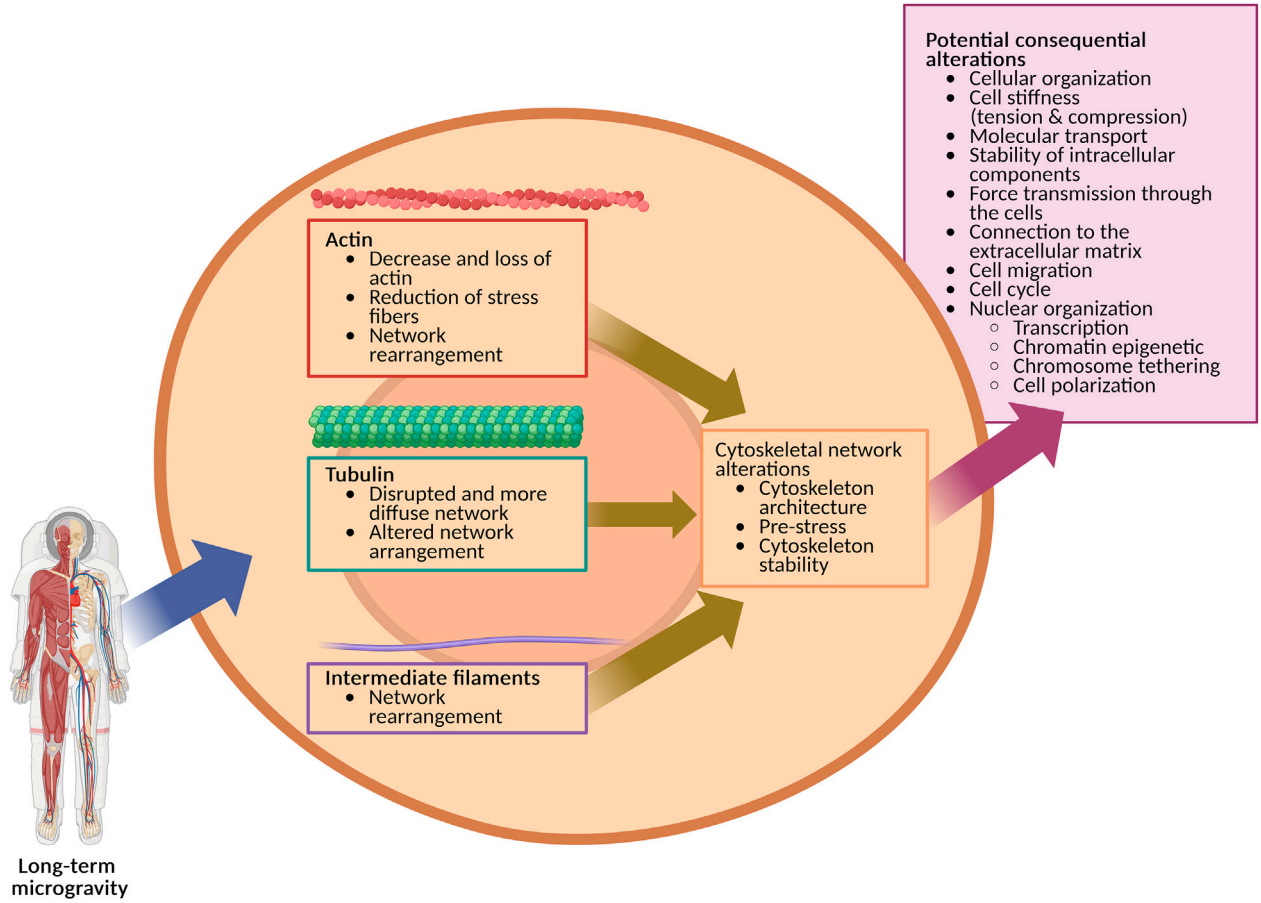

FIGURE 4
Long-term microgravity impacts the cytoskeleton's organization, which could result in multiple subsequent cellular alterations (Created in BioRender. https://BioRender.com/gt4daok).

gravity could act as an "organizing force" supporting an efficient buildup and organization of the cytoskeleton. This perspective is backed by the early experiments which demonstrated that the self-assembly of microtubules in a cell-free system is indeed gravity dependent (Moos et al., 1988; Papaseit et al., 2000). As the entire evolution developed in the Earth's constant gravity condition, even molecular processes are likely fine-tuned to work in a gravity field. Traditional "fix and rinse experiments" are relatively easy and cost-effective to implement but suffer from limited time points and large data variability (unpaired data). In contrast, life cell staining techniques (Corydon et al., 2016; Nassef et al., 2019) allow observing changes in real-time over extended periods with high special and temporal resolution. In combination with pharmaceutical blockers or genetic manipulations (e.g. knockout or knock-in) this technology could help to mechanistically dissect the underlying molecular pathways.

In conclusion, understanding and quantifying the effect of microgravity on the cytoskeleton and its potential implications is not trivial. The body of literature from the past decades does not always show consistent patterns and is sometimes even contradictory. Generally speaking, the time cells are exposed to microgravity seems to be the dominant contributor to trigger clearly observable modifications.

4 Methods

4.1 Score determination

First a thorough literature search was conducted, and the publications were manually screened. Scientific publications reporting cytoskeleton adaptions in real microgravity, using immunofluorescent imaging, were included. (A few potential hits could not be included, because the full text was not accessible.) The results of 30 included publications were subsequently compiled and rated by ten individuals. For each claim by the original author, the allowed scores were "no" change (0), "small" (barely visible; 1), "fair" (clearly visible; 2) or "strong" (obvious; 3) change. All information regarding the authors, research institutions, utilized microgravity platform or cell source was removed to avoid biasing the scoring. The raters were not familiar with the publications, and all had a master or PhD in the biomedical field and were familiar with cytoskeleton staining. Publications in which researchers investigated multiple proteins (e.g., actin and tubulin) or time points were split, and the proteins and timepoints were scored separately. Publications that reported no change were not displayed for scoring and directly scored as showing "no" change.

TABLE 4 Resulting average scores were classified into seven categories.

Score classification	Score number range
Strong	2.9. . .3.0
Fair to strong	2.2. . .2.8
Fair	1.9. . .2.1
Small to fair	1.2. . .1.8
Small	0.9. . .1.1
No to small	0.2. . .0.8
No	0.0. . .0.1

To compare the studies (Figures 1–3), for each protein and timepoint the largest score given by an individual rater was taken and then averaged over all ten raters for the final score. To assess the individually reported effects, for each author's claim, the scores were averaged over the ten raters and ranked from "strong" effect to "no" effect. The resulting average scores were finally classified according to Table 4. The 95% confidence interval (CI) is the bootstrap CI of the mean value.

4.2 Time dependency

Time dependency was calculated by the single-tailed Pearson correlation coefficient of the log-transformed time versus the averaged score per publication and timepoint. Pearson's ρ and p-values are reported in Supplementary Table S1.

Author contributions

SW: Investigation, Writing – review and editing, Conceptualization, Writing – original draft, Data curation, Visualization, Methodology.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frspt.2025.1677728/full#supplementary-material

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