



**CONCISE REVIEW**

# The effects of microgravity on differentiation and cell growth in stem cells and cancer stem cells

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**Abstract**

A spaceflight has enormous influence on the health of space voyagers due to the combined effects of microgravity and cosmic radiation. Known effects of microgravity ( $\mu\text{g}$ ) on cells are changes in differentiation and growth. Considering the commercialization of spaceflight, future space exploration, and long-term manned flights, research focusing on differentiation and growth of stem cells and cancer cells exposed to real (r-) and simulated (s-)  $\mu\text{g}$  is of high interest for regenerative medicine and cancer research. In this review, we focus on platforms to study r- and s- $\mu\text{g}$  as well as the impact of  $\mu\text{g}$  on cancer stem cells in the field of gastrointestinal cancer, lung cancer, and osteosarcoma. Moreover, we review the current knowledge of different types of stem cells exposed to  $\mu\text{g}$  conditions with regard to differentiation and engineering of cartilage, bone, vasculature, heart, skin, and liver constructs.

**KEYWORDS**

cancer stem cells, microgravity, multicellular spheroids, organoids, random positioning machine, rotating wall vessel, spaceflight, stem cells, tissue engineering

**1 | INTRODUCTION**

Microgravity ( $\mu\text{g}$ ) induces a large number of changes in specialized cells and stem cells.<sup>1-3</sup> Experiments in orbit on the International Space Station (ISS), on unmanned spacecrafts or sounding rockets and on Earth using devices simulating  $\mu\text{g}$  such as the random positioning machine (RPM), or a clinostat

(CN) demonstrated among others alterations of the cytoskeleton,<sup>4-7</sup> changes in the composition of the extracellular matrix,<sup>8</sup> focal adherence complex,<sup>6</sup> proliferation,<sup>9</sup> differentiation,<sup>10</sup> and growth behavior of the cells.<sup>1</sup> These changes were observed in different cell types.<sup>1</sup>

Gravitational biology and space medicine are currently of high interest and a hot topic in space research. A PubMed search on

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27 February 2020 gave 9010 matches for the term “weightlessness,” 11 338 matches for “microgravity,” 549 matches for “microgravity and differentiation,” and 273 matches for “microgravity and stem cells.”

An important aspect is the differentiation and redifferentiation potential of benign and malignant cells grown under simulated and real  $\mu\text{g}$  conditions. Endothelial progenitor cells exposed to  $\mu\text{g}$  revealed an improved angiogenic potential.<sup>11</sup> Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) cultured aboard the ISS exhibited alterations in calcium handling and showed 2635 differentially expressed genes among flight, postflight, and ground control samples.<sup>12</sup> In particular, genes involved in mitochondrial metabolism were differentially regulated.<sup>12</sup> Recently, human blood-derived stem cells had been investigated on the ISS. Compared with cells cultivated on Earth, a reduced expression of Sox2, Oct3/4, Nanog, and E-cadherin was measured in space, together with an earlier osteogenic differentiation.<sup>13</sup> In addition, cancer cells have proven to re-differentiate after exposure to r- and s- $\mu\text{g}$ .<sup>10,14,15</sup> Studies in  $\mu\text{g}$  demonstrated that cells exposed to gravitational unloading changed their growth behavior and started growing in a two- (2D) and three-dimensional (3D) manner in space and on the rotating wall vessel (RWV), the RPM, CN, or on rotary cell culture systems (RCCS) (Figure 1).<sup>1,16-20</sup>

Thus, it is possible to engineer scaffold-free and scaffold-containing 3D tissues such as preliminary vessels, cartilage, liver and bone pieces, and other organoids from different cell types using  $\mu\text{g}$ .<sup>1</sup> Currently, a large number of publications reported about the behavior of cancer cells in  $\mu\text{g}$ , but little information exists about the behavior of cancer stem cells (CSCs) in  $\mu\text{g}$ . Recently, investigations on colorectal CSCs were possible when HCT116 cells were exposed to a RCCS. Microgravity conditions provide a new method to study the biology of cancer stemness.<sup>14</sup>

This concise review will focus on the current knowledge of the impact of  $\mu\text{g}$  on differentiation and cell growth in stem cells and CSCs.

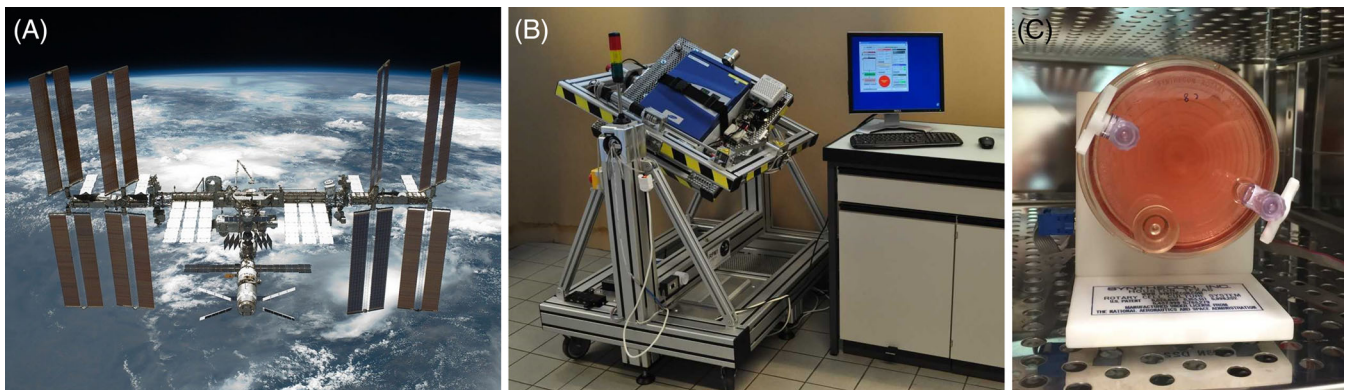
## 2 | DEFINITION OF CSCs

CSCs represent a subset of cells within the heterogeneous bulk of liquid and solid tumors.<sup>21</sup> The term CSC was introduced for the first

### Significance statement

Microgravity provides a unique environment for cell culture and has been shown to induce cellular changes and processes that could not be achieved under normal gravitational conditions. Over the past years, it has therefore gained increasing importance in different research fields such as cancer research, where microgravity may help understanding and suppressing tumor metastasis, or tissue engineering, where it induces the scaffold-free formation of three-dimensional multicellular spheroids. This review will give a concise overview of the current knowledge on the effects of microgravity on stem cells and cancer stem cells, and will highlight novel therapeutic options derived from it.

time by Dr John E. Dick and colleagues more than 25 years ago in their revolutionary study of certain kinds of human leukemia.<sup>22</sup> CSCs share features with somatic stem cells, and therefore they possess characteristics of asymmetric division, self-renewal, quiescence, and differentiation.<sup>23</sup> In addition, these cells own the ability to replicate the parental tumor upon transplantation into a host as shown by Dick et al.<sup>22</sup> CSCs also play a pivotal role in tumor pharmacological resistance due to upregulation of drug efflux transporters and DNA repair components.<sup>21,23</sup> Besides leukemia, CSCs have been identified in numerous tumor types, including those of the brain, breast, prostate, kidney, lung, and colon.<sup>23,24</sup> Accumulating evidence suggests mutations and epigenetic alterations in some tumor cells as main drivers leading to the establishment of CSCs.<sup>25</sup> Microenvironment-driven selection has also been implicated in the emergency of resistant cells with stem-like characteristics.<sup>26</sup> As CSCs are highly resistant to current therapeutic approaches, and therefore the main reason for cancer relapse, these cells are considered the prime therapeutic target for cancer treatment.<sup>25,27-29</sup>



**FIGURE 1** Platforms and devices used for stem cell research under real and simulated microgravity conditions. A, The International Space Station enables long-term cell culture in real microgravity. B, Custom-made random positioning machine (RPM) for the cultivation of mammalian cells under simulated microgravity conditions.<sup>20</sup> C, The rotating wall vessel (RWV) is another type of microgravity simulator suitable for suspension cell cultures or adherent cells on microcarrier beads

### 3 | CSCs EXPOSED TO MICROGRAVITY

Little is known about the behavior of CSCs exposed to  $\mu\text{g}$  conditions, but cancer researchers have started to work on this important topic.

Tumor cell heterogeneity and the presence of CSCs are responsible for a poor outcome of cancer patients and that metastatic breast cancer remains currently incurable, despite the progress in their detection and treatment.<sup>30</sup> Breast CSCs have been identified as CD44<sup>high</sup>, CD24<sup>low</sup>, or aldehyde dehydrogenase positive (ALDH<sup>+</sup>).<sup>31,32</sup> Breast, thyroid, and prostate cancer cells differentiate and show various morphological alterations, change their growth behavior when grown in space or under  $s\text{-}\mu\text{g}$ .<sup>33-36</sup> The cancer cells differentiate into two phenotypes in the  $\mu\text{g}$  environment. One part grows adherently on the bottom of the cell culture flasks and the other one assembled to 3D spheroids.<sup>33,34,36,37</sup> Multicellular spheroids (MCS) represent an intermediate metastasis model in its complexity between a monolayer culture and the in vivo tumor. These 3D MCS have the features of the primary tumor and also exhibit stem-like features.<sup>38</sup> Therefore,  $\mu\text{g}$ -engineered spheroids of different cancer types may represent an excellent model for detailed CSCs research.

Focused research on CSCs exposed to  $\mu\text{g}$  is published for various types of tumors.

#### 3.1 | Lung cancer

As in most other tumors, lung CSCs exhibit an increased expression of cell surface markers CD44 and CD133. Alternatively, lung CSCs may be purified using functional assays like ALDH<sup>+</sup><sup>8</sup> and side population (SP) assays.<sup>39</sup> Several studies have investigated the influence of reduced gravity on lung cancer cell behavior.<sup>40-42</sup> However, only few studies, to our knowledge, have reported an impact of  $s\text{-}\mu\text{g}$  on lung CSCs. One prominent example is the study by Pisanu et al in which lung CSCs, enriched from the stable non-small cell lung cancer cell line H460, were subjected to  $s\text{-}\mu\text{g}$  obtained by means of an RPM.<sup>15</sup> The study provides evidence that lung CSCs are committed to selective differentiation when subjected to reduced gravity. Furthermore, the study also reported increased apoptosis of CSCs incubated on the RPM compared to 1g controls. Collectively, these data suggest that lung CSCs, similar to somatic stem cells, are rescued from their quiescent state and lose their stemness default state when cultured in  $\mu\text{g}$ . Consistent with this observation, a decrease in ALDH levels as well as downregulation of *Nanog* and *Oct4* genes were observed. Intriguingly, the  $\mu\text{g}$ -induced traits were stably attained and conserved by CSCs when cells were returned to 1g conditions.<sup>15</sup>

#### 3.2 | Gastrointestinal tumors

The gastrointestinal tract comprises stomach, colon, small intestine, rectum, and pancreas. During embryogenesis, these organs develop from stem cells and even in adults relevant stem or progenitor cells are persisting.<sup>43</sup> They are capable to keep up the integrity of these organs, but may also be the origin of cancer development.<sup>44</sup>

Therefore, stem cell behavior is intensively investigated by a number of methods including exposure to  $s\text{-}$  or  $r\text{-}\mu\text{g}$ .<sup>45</sup>

However, little is known about the  $\mu\text{g}$ -dependent behavior of stem cells of the gastrointestinal tract. Whether the downregulation of the tropomyosin 1 gene (*Tpm1*) in stem cells<sup>46</sup> is responsible for downregulated *Tpm1* gene in gastrocnemius muscle of mice observed after a 12-day lasting spaceflight remains to be proven.<sup>47</sup> Until now, Arun et al published a report about CSCs included in a population of colorectal cancer HTC116 cells.<sup>48</sup> They cultured the whole population on a RCCS and observed that the percentage of CSCs expressing CD133 and CD44 simultaneously increased within the cell population. Their work suggests that  $\mu\text{g}$  affects the growth/differentiation control elements FOXO3/PTEN/AKT.<sup>14</sup> In addition, Devarasethy et al used an RWV bioreactor to form tumor organoids consisting of human hepatocytes, mesenchymal stem cells (MSCs) and colon carcinoma HCT116 cells. The subsequent investigation of the organoids revealed that the ratio of stem cells influenced the growth rate of the colon carcinoma cells.<sup>49</sup>

Stem cells are also present within adult human and mice pancreas cell populations.<sup>50,51</sup> If pancreatic cells are singularized and cultured within a hanging drop culture, they form pseudoislets with improved physiological properties.<sup>52</sup> The result could be explained by the hypothesis that 3D spheroid formation favors the growth of progenitors, as it has been shown by Samuelson and Gerber,<sup>53</sup> who successfully induced differentiation of mouse pancreatic progenitor cells by culturing them in a RWV bioreactor. Furthermore, if human pancreatic islets were predispersed and cocultured with stem cell such as amniotic epithelial cells (AECs) on a rotational cell culture system, islet:AEC aggregates were formed with improved insulin secretory capacity.<sup>54</sup>

#### 3.3 | Osteosarcoma and other tumors

Kelly et al investigated human osteosarcoma cells (SAOS-2 cells) exposed to the NASA-developed hydrofocusing bioreactor (HFB) and to the RCCS.<sup>55</sup> The authors showed that CSCs were stimulated to proliferate when they are cultured in  $\mu\text{g}$  conditions produced by the HFB. In addition,  $\mu\text{g}$  sensitized CSCs to chemotherapeutic agents.<sup>55</sup> Moreover, the authors investigated various cell types on the HFB such as osteosarcoma cells (SAOS-2), prostate cancer cells, lung cancer cells, and melanoma cells and demonstrated that HFB exposure increased CD133-positive cell growth from various cell lines compared with the RCCS vessel and to normal gravity control.<sup>55</sup>

## 4 | MICROGRAVITY INFLUENCES DIFFERENTIATION AND GROWTH BEHAVIOR OF STEM CELLS

### 4.1 | Use of stem cells for cartilage tissue engineering

Considering the overall development of progressively aging societies, the impact of cartilage damage and accompanying disorders such as

osteoarthritis, rheumatoid arthritis, or intervertebral disk damage is steadily growing. One main problem of the treatment of cartilage lesions is the poor regenerative capacity of this tissue due to its avascularity and relatively low cell density, which is further augmented by the fact that the surviving chondrocytes shift their metabolic activity toward predominantly degenerative processes.<sup>56</sup> For applications in regenerative medicine, it is therefore of high interest to circumvent these problems and to stimulate cartilage repair. One approach for this is the use of stem cells (Table 1).

MSCs derived either from bone marrow or from adipose tissue are most widely used. In general,  $s\text{-}\mu\text{g}$  seemed to be beneficial in promoting the differentiation of MSC into a chondrogenic phenotype. Ohyabu et al observed large tissue constructs after a 4-week culture of rabbit MSCs in an RWV bioreactor. Their cartilaginous nature was confirmed by qPCR analyses of aggrecan and collagen types I and II gene expression as well as by toluidine blue and safranin-O staining.<sup>57</sup> Other authors described similar findings, where cultivation of MSCs under  $s\text{-}\mu\text{g}$  conditions led to a higher expression of chondrogenic markers than in static control groups and to a better quality cartilage tissue compared with standard 3D culture techniques.<sup>58-60</sup> It has been suggested that these effects are mediated via the p38 MAPK pathway.<sup>61</sup> Furthermore, simulated  $\mu\text{g}$  was also reported to potentiate the proliferative capacity of MSCs.<sup>60</sup>

Yin et al have introduced the use of decellularized cartilage extracellular matrix-derived particles as scaffolds for the culture of rabbit adipose-derived stem cells as well as bone marrow stromal cells and found that  $s\text{-}\mu\text{g}$  conditions greatly improved the induction of stem cell chondrogenesis as well as in vivo repair of cartilage lesions in an animal model.<sup>62,63</sup> Another coculture approach used primary meniscus cells and MSCs.  $s\text{-}\mu\text{g}$  strongly increased cartilage matrix formation, the expression of hypertrophic differentiation markers COL10A1 and MMP-13, and suppressed the hypertrophic differentiation inhibitor, gremlin 1.<sup>64</sup> Interestingly, a recent study with Indian hedgehog and Sonic hedgehog transfected MSCs revealed that  $s\text{-}\mu\text{g}$  significantly promoted the differentiation of MSCs into chondrocytes and also inhibited chondrocyte hypertrophy and aging during chondrogenesis.<sup>65</sup>

It should be noted, however, that not all authors have found a promoting effect of  $s\text{-}\mu\text{g}$  on the chondrogenic differentiation of MSCs. In their experiments exposing human MSCs to  $s\text{-}\mu\text{g}$ , Mayer-Wagner et al observed a lowered hypertrophy, but also a reduced chondrogenic potential, which could be in part counteracted by a low-frequency electromagnetic field,<sup>66,67</sup> emphasizing the necessity for further research in this promising field.

## 4.2 | Stem cells for the engineering of bone-like tissues

It is well known that  $\mu\text{g}$  influences calcium, sodium, and bone metabolism of humans in space. Bone loss and spaceflight-induced osteopenia are problematic issues for long-term space missions and future space exploration.<sup>68</sup> Bone loss and radiation are the most substantial health risks of astronauts and should be managed by efficient countermeasures.<sup>68</sup>

Despite this knowledge from space travelers, it is also well acknowledged that culturing of stem cells and bone cells in  $s\text{-}\mu\text{g}$ , using the RWV, the RCCS bioreactor, or the RPM, is a suitable technique for bone tissue engineering purposes<sup>69-71</sup> and has been reviewed earlier (Table 1).<sup>1,3</sup> 3D cell culture environments were useful to enhance osteoblast differentiation and to engineer osseous-like tissues from human preosteoblastic cells and human mesenchymal preosteoblastic cells (Figure 2).<sup>69,70</sup> The RWV bioreactor was used and patented by Clarke et al to grow mineralized 3D bone constructs.<sup>72</sup> Another study demonstrated that after a 2-week culture of fetal osteoblasts (hFOB 1.19 cell line) on an RPM, MSCs could be engineered presenting a bone-specific morphology. The hFOB 1.19 cells are well-characterized, stable osteoprogenitor and a well-known model for normal osteoblast differentiation.<sup>71</sup> These bone tissues may be used to study the mechanisms behind spaceflight-related bone loss or other bone diseases such as osteonecrosis or bone injuries.<sup>71</sup>

A recent study demonstrated the osteogenic potential of rat bone marrow-derived MSCs (BMSCs) in vitro.<sup>73</sup> Encapsulated BMSCs differentiated into osteoblastic cells and had formed bone-like tissue under osteogenic  $\mu\text{g}$  bioreactor conditions.<sup>73</sup>

Human blood-derived stem cells (BDSCs) were investigated recently in space on the ISS during the Italian VITA mission. Osteoblastic differentiation was induced by rapamycin.<sup>13</sup> Rapamycin influenced the transcriptional activation of BDSCs toward osteogenic differentiation via elevated GATA4 and Sox17. Both factors modulate downstream transcription factors (like Runx2), critical for bone formation.<sup>13</sup>

Another study reported on the behavior of human BMSCs exposed to  $s\text{-}\mu\text{g}$ . The authors found an inhibition of proliferation and differentiation toward osteoblasts, but an increase in adipogenesis.<sup>74</sup>  $s\text{-}\mu\text{g}$  also selected highly tumorigenic cells for survival.<sup>74</sup> In addition, MSCs cultured on the SJ-10 recoverable scientific satellite revealed an increase in the p38 MAPK activity and a de-repression of AKT activity. The satellite flight conditions inhibited osteogenic differentiation and promoted adipogenic differentiation, even under osteogenic induction conditions.<sup>75</sup>

Bone MSCs exposed to  $\mu\text{g}$  revealed alterations in regulation or functioning of the actin cytoskeleton which may cause the inhibited osteogenesis.<sup>76</sup> The depolymerization of actin inhibited the osteogenic differentiation of the bone MSCs through impeding nuclear aggregation of the transcriptional coactivator with PDZ-binding motif.<sup>76</sup> Bradamante et al investigated human bone marrow stem cells, which were cultivated on the ISS for 14 days. They used vitamin D<sub>3</sub> as an osteogenic differentiation inducer and compared the gene expression of in-flight and on-ground samples. They found that  $\mu\text{g}$  had mainly an effect on the composition of the extracellular matrix by reducing collagens among other, while apoptosis was absent.<sup>77</sup>

## 4.3 | Stem cells for vasculature and heart tissue engineering

### 4.3.1 | Vasculature

Culturing immature endothelial stem cells (ESCs) or progenitor cells (EPCs) under  $\mu\text{g}$  conditions provide a valuable tool for therapeutic

**TABLE 1** Summary of selected articles addressing research on cancer stem cells and stem in vitro cultured under simulated microgravity, ordered by organ/tissue

Cell line	Organ/tissue/cell type	Space or $\mu$ g-simulating device	Findings	References
H460	Non-small cell lung cancer	RPM	Increase in apoptosis, CSCs lost their stemness features, downregulated <i>Nanog</i> and <i>Oct4</i> genes	Pisanu et al <sup>15</sup>
HCT116	Colorectal cancer	RCCS	CSC; CD133/CD44 dual positive cells, giant cancer cells housing complete nuclear localization of YAP	Arun et al <sup>14,48</sup>
SAOS-2, HOS, U2OS T98G, U87MG Du145, LNCap H23 Hep3b Hela Mewo, HO-1	Osteosarcoma Glioblastoma Prostate cancer Lung cancer Hepatocarcinoma Cervical carcinoma Melanoma	HFB	CD133 <sup>+</sup> cells from cancer cell lines	Kelly et al <sup>55</sup>
Rabbit MSCs	Cartilage tissue constructs	RWV	Cartilage nature confirmed by aggrecan and collagen types I and II gene expression as well as by toluidine blue and safranin-O staining	Ohyabu et al <sup>57</sup>
Rabbit adipose-derived stem cells and bone marrow stromal cells	Cartilage	RCCS, novel cell carrier derived from natural cartilage ECM	Improved the induction of stem cell chondro-genesis as well as in vivo repair of cartilage lesions in a rabbit model	Yin H et al <sup>62,63</sup>
hMSCs	Bone marrow, osteogenic lineage	RWV	Not suitable for a potential application in cartilage repair	Mayer-Wagner et al <sup>66</sup>
hBMSCs	Bone marrow	ISS	r- $\mu$ g stresses reverting to a quiescent state	Bradamante et al <sup>77</sup>
hADMSCs	Adipose tissue	RPM	Oxygen is a key player for cytoskeletal alterations and modulation of gene expression	Versari et al <sup>118</sup>
Rat BMSCs	Bone-like tissue	STLV bioreactor, chitosan/hydroxyapatite, 28 days	BM-MSCs-C/HAP composite microbeads	Koç Demir et al <sup>73</sup>
BMSCs	Bone marrow	Clinostat	Depolymerized actin cytoskeleton inhibits osteogenic differentiation of BMSCs through impeding nuclear aggregation of TAZ	Chen et al <sup>76</sup>
CD34-positive human cord blood stem cells (CBSC)	Vascular tubular assemblies	RWV, with or without Cytodex-3 microcarrier beads and VEGF	Transdifferentiation into the vascular endothelial cell phenotype and assembling into 3D tissue structures	Chiu et al <sup>80</sup>
BMSCs	Endothelium-like cells	Clinostat	Endothelial-specific molecules (Flk-1 and vWF) positive	Zhang et al <sup>82</sup>
EPCs	PBMNC	3D clinostat	Most significant increase in CD34 <sup>+</sup> and double positive Dil-Ac-LDL-FITC-Ulex-Lectin cells, both EPC markers. Enhancing the number and angiogenic potential of EPCs	Hagiwara et al <sup>9</sup>
Pluripotent stem cell-derived cardiomyocytes	Heart	ISS	Alterations in hiPSC-CM calcium handling showed 2635 differentially expressed genes	Wnorowski et al <sup>12</sup>



**TABLE 1** (Continued)

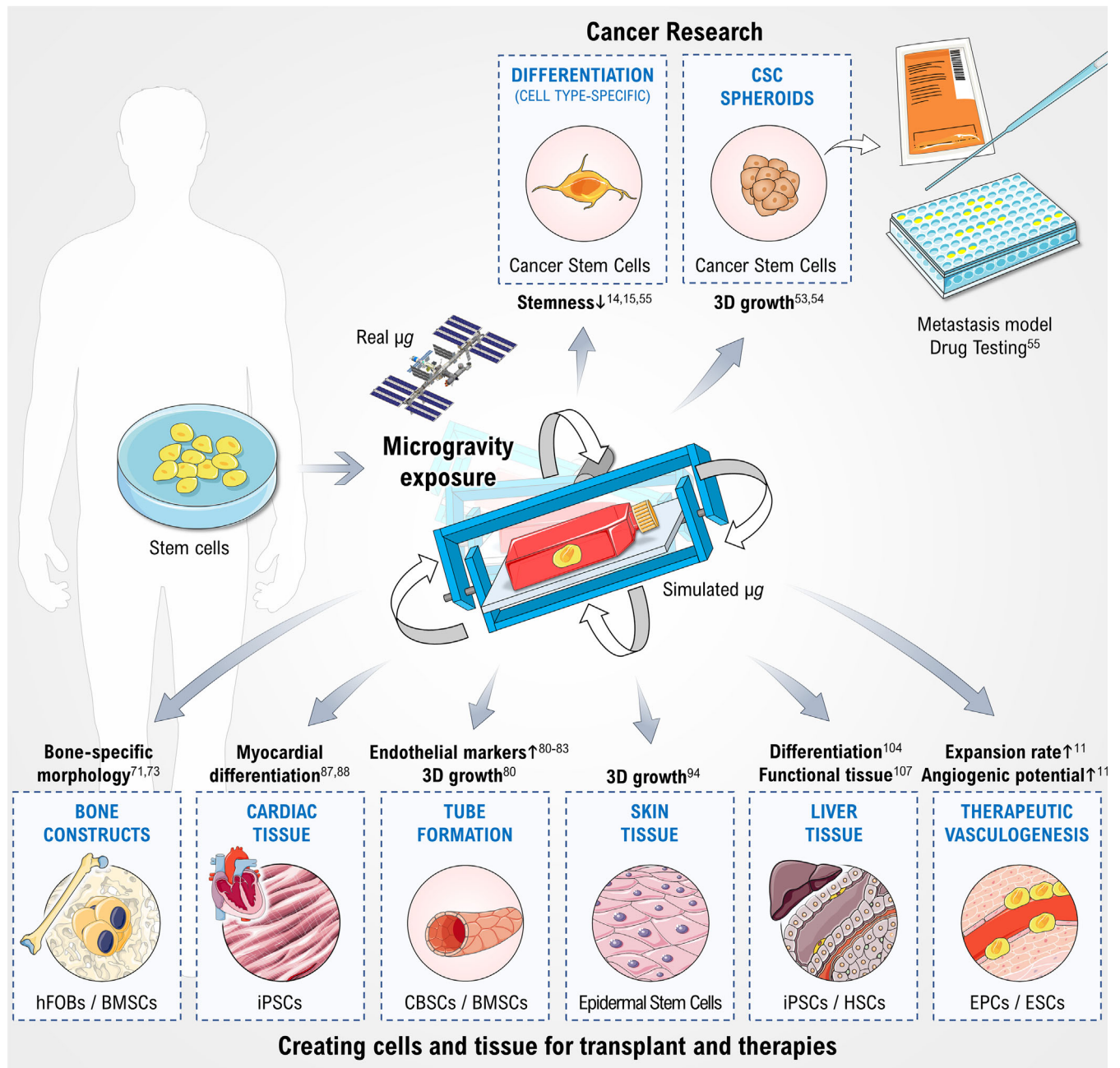
Cell line	Organ/tissue/cell type	Space or $\mu$ g-simulating device	Findings	References
CPCs	Cardiac tissue	ISS, 2D Clinostat	Hippo signaling; upregulation of downstream genes: <i>YAP1</i> and <i>SOD2</i>	Camberos et al <sup>85</sup>
Adult and neonatal CPCs	Cardiac repair	ISS	Only neonatal CPCs showed an increased expression of early developmental markers and an enhanced proliferative potential	Baio et al <sup>84</sup>
IMR90 iPSCs, hESCs (H7 and H9)	Progenitor cardiac spheres	RPM	Progenitor cardiac spheres (RPM) result in efficient generation of highly enriched hPSC-CMs. Increase in proliferation and viability of CPCs	Jha et al <sup>89</sup>
Mouse ESCs	Mouse embryo	2D Clinostat	Deregulation of genes involved in heart development and inhibition of cardiomyocyte specific genes	Shinde et al <sup>46</sup>
PICM-19	Pig liver tissue	Spaceflight, STS-126 mission	In vitro model for assessing liver function in $\mu$ g. Minor differences between 1g and $\mu$ g	Talbot et al <sup>109</sup>
ADSCs	Subcutaneous adipose tissue	$\mu$ g bioreactor	Stemness properties, including self-renewal and multipotency differentiation capacities, were enhanced by spheroid formation in $\mu$ g. Spheroid-derived ADSCs showed more effective potentials to rescue liver failure	Zhang et al <sup>108</sup>
HepG2 Human biliary tree stem/progenitor cells (hBTSCs)	Hepatocyte carcinoma	RCCS	s- $\mu$ g promotes the formation of 3D cultures and stimulates pluripotency and glycolytic metabolism in human hepatic and biliary tree stem/progenitor cells	Costantini et al <sup>105</sup>
Human epidermal stem cells (hEpSCs)	Epidermis-like structure	RWV bioreactor, Cytodex-3 microcarriers	hEpSCs aggregated on the microcarriers and formed multilayer 3D epidermis structures	Lei et al <sup>94</sup>
ADSCs	Adipose tissue	2D clinostat, CTGF	Differentiation to fibroblast cells. <i>Col1</i> and <i>ColIII</i> , <i>MMP1</i> , <i>ITGB1</i> , and <i>FSP1</i> gene expression changes involved	Ebnerasuly et al <sup>95</sup>

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; AD(M)SCs, adipose tissue-derived (mesenchymal) stem cells; BMSCs, bone marrow mesenchymal stem cells; *Col1*, collagen type I gene; *ColIII*, collagen type III gene; CPCs, cardiac progenitor cells; CTGF, connective tissue growth factor; ESCs, embryonic stem cells; EPCs, endothelial progenitor cells; FLK1, fetal liver kinase 1; *FSP1*, fibroblast-specific protein 1 gene; hBTSCs, human biliary tree stem/progenitor cells; HFB, hydrodynamic focusing bioreactor; MCS, multicellular spheroids; *MMP1*, matrix metalloproteinase 1 gene; PBMNCs, peripheral blood mononuclear cells; RCCS, rotary cell culture system; RPM, random positioning machine; RWV, rotating wall vessel; r- $\mu$ g, real microgravity; s- $\mu$ g, simulated microgravity; *SOD2*, superoxide dismutase 2; STLV, slow turning lateral vessel-type rotating bioreactor; TAZ, PDZ-binding motif; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor; *YAP1*, yes-associated protein 1 gene.

vasculogenesis and tissue regeneration and may contribute to the steadily and rapidly progressing discipline regenerative medicine. For example, EPC transplantation has become beneficial for ischemic diseases.<sup>78</sup> However, the deficiency of functional EPCs in adults reflects the limiting factor for EPC transplantation as a neovascularization therapy. Microgravity was shown to improve the numbers of hematopoietic progenitor cells<sup>79</sup> as well as the functions of stem cells.<sup>60</sup> Hagiwara et al recently demonstrated that an initial cultivation of EPCs in  $\mu$ g followed by cultivation under normal gravity remarkably

enhanced expansion rates and angiogenic potential, including vascular endothelial growth factor (VEGF).<sup>11</sup>

Microgravity provides new possibilities for cell-based therapy (reviewed by Imura et al<sup>45</sup>). In 2005, Chiu and colleagues cultured CD34-positive human cord blood stem cells (CBSCs), which are involved in vascularization, in a RWV bioreactor together with or without Cytodex-3 microcarrier beads and supplemented them with VEGF.<sup>80</sup> After 4 days, the cells cultured without carrier beads assembled to tubular structures (TS) and expressed endothelial phenotypic markers such as



**FIGURE 2** Effects of real or simulated microgravity on stem cell cultures (lower panel) and cancer stem cells (upper panel) together with the possible applications in medicine. Arrows indicate increases/reductions. BMSC, bone marrow-derived stem cell; CBSC, umbilical cord blood stem cell; CSC, cancer stem cell; EPC, endothelial progenitor cell; ESC, endothelial stem cell; hFOB, human fetal osteoblast; HSC, hepatic stem cell; iPSC, induced pluripotent stem cell;  $\mu g$ , microgravity. Parts of the figure are drawn using pictures from Servier Medical Art (<https://smart.servier.com>), licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0>)

CD31 and kinase insert domain receptor (KDR, former: Flk1), whereas only amorphous cell clusters were formed in the presence of the beads. This experiment proved that CBSCs can differentiate into a vascular endothelial phenotype under  $\mu g$  conditions and are able to assemble into TS.

Furthermore, the  $\mu g$  environment increases the multipotential differentiation capacity of BMSCs. Endothelium-oriented differentiated BMSCs have been shown to express higher levels of von Willebrand factor (VWF) and CD31.<sup>81</sup> Zhang et al described the differentiation of BMSCs into endothelial-like cells after 72 hours of clinorotation. Cells

expressed the endothelial-specific markers KDR and VWF and were able to form a capillary network.<sup>82</sup>

Interestingly,  $\mu g$  may stimulate cardiovascular progenitors differently depending on their age. Whereas adult cells showed elevated expression of endothelial markers and revealed unchanged or increased endothelial cell tube formation, neonatal cells showed a decline in tube formation and acquired characteristics of dedifferentiation after 6 to 7 days of clinorotation.<sup>83</sup> Aboard the ISS, cytoskeletal organization, migration, and expression of DNA repair genes were

affected both in neonatal and adult cardiovascular progenitors. However, only neonatal cells showed enhanced developmental markers and proliferative potential.<sup>84</sup> RNA-sequencing revealed that 2635 genes were differentially expressed among  $\mu\text{g}$ -exposed and control samples, including genes involved in mitochondrial metabolism.<sup>12</sup> In addition, adult cardiovascular progenitors cultured under different  $\mu\text{g}$  conditions upregulated downstream genes involved in the Hippo pathway, such as *YAP1* and *SOD2*, which may have potential benefit for cardiovascular repair.<sup>85</sup>

### 4.3.2 | Heart tissue

Biofabrication of heart tissue is a hot topic in tissue engineering. The usefulness of tissue engineered cardiac tissue is dependent among others on the differentiation status of cardiomyocytes (CMs) *in vitro*, on the contractility, and electrophysiological characteristics.<sup>86</sup> As the growth of CMs is in part regulated by mechanical loading, the idea suggests itself that  $\mu\text{g}$  can lead to growth changes as well. A recent study on the ISS investigated human-induced pluripotent stem cell-derived CMs.<sup>10</sup> The authors demonstrated that long-term cell culture (5.5 weeks) of advanced, highly specialized cell types such as human CMs is possible aboard the ISS.

Microgravity has proven to alter the CM behavior and thus can help to promote myocardial differentiation of induced pluripotent stem cells.<sup>87,88</sup> A combination of 3D culture and  $s\text{-}\mu\text{g}$  created by an RPM can be used to efficiently generate highly enriched CMs.<sup>89</sup> Dissociated cardiac muscle cells cultured on 3D scaffolds in RCCS formed engineered constructs with structural and functional features resembling those of native cardiac tissue.<sup>90</sup> Another study reported that simulated  $\mu\text{g}$  provided by a 2D CN modulated the differentiation processes of embryonic stem cells.<sup>46</sup> This ability can be a driving element for biofabrication methods.

In view of future space exploration plans, it is of high interest to test the effects of simulated Mars and Moon gravity using a special RPM manufactured by ADS, Leiden, and NL on the tissue engineering of heart tissue.<sup>91</sup>

### 4.4 | Stem cells for skin repair

Different resident skin stem cell pools contribute to the maintenance and repair of the various epidermal tissues of the skin. Their development to differentiated skin cells is regulated by various factors.<sup>92</sup> There are a few publications, which suggest that  $\mu\text{g}$  may have an influence on this process. Exposing murine embryonic stem cells to parabolic flights changed 14 genes, which are involved in skin development.<sup>93</sup>

Human epidermal stem cells (hEpSCs) isolated from children's foreskin were successfully propagated *in vitro*, while hEpSCs marker and proliferative capacity are maintained. Subsequently, they were seeded in a rotary bioreactor together with microcarrier beads for 1 day, which allowed the cells to attach to the surface of the beads.

Then, the sample was divided into two groups, including a rotation culture group in an RCCS and a static culture group on six-well cell culture plates. The cells in a rotary bioreactor were prone to accumulate on the microcarrier beads forming 3D aggregates, whereas the cells in six-well cell culture plates appeared as monolayer sheet structure on the surface of microcarrier beads. In the rotation culture group, there were a large number of cells aggregated over microcarriers beads forming 3D tissue-like epidermis structure.<sup>94</sup> Fibroblasts, an important type of skin cells, could be derived from adipose-derived stem cells (ADSCs) exposed to  $\mu\text{g}$ . They were isolated from fat specimens of patients. Afterward, they were cultured on a 2D CN and under 1g conditions. Comparing the cells after 7 days of incubation showed that the connective tissue growth factor-induced fibroblastic differentiation of ADSCs can be modulated by exposure to  $s\text{-}\mu\text{g}$ . Most notably, the expression of CD44 was reduced while the production of collagen III was enhanced.<sup>95</sup>

### 4.5 | Use of stem cells for the engineering of liver tissue

The liver is the largest inner organ and exhibits numerous unique biochemical functions. More than 500 physiological functions of the liver are reported.<sup>96,97</sup> The liver is involved in metabolic processes, in biochemical synthesis as well as in detoxification processes. Although it shows a high capacity of regeneration, diseases of the liver such as cirrhosis, hepatitis, and hepatocellular carcinomas account for about 2 million deaths per year worldwide.<sup>98</sup> Exposure of the liver to toxicants such as alcohol due to alcohol misuse or infection with hepatitis C and B viruses and other nonalcoholic liver diseases<sup>99</sup> may lead to liver cirrhosis, which is responsible for about 1.03 million deaths per year.<sup>100</sup> Up to now, orthologous liver transplantation is the only treatment option available for patients suffering from such severe liver diseases. However, there is a drastic shortage of donor organs,<sup>98,101,102</sup> so alternative methods for treating end-stage liver disease and liver regeneration should be explored.

Majumder et al showed that only 2 hours of incubation of bipotential murine oval liver stem cell under  $s\text{-}\mu\text{g}$  on a 3D CN were sufficient to enhance their proliferation 2-fold and to induce differentiation into hepatocytes within 2 to 3 days, a process probably driven by an interplay of  $\mu\text{g}$  with BMP4/Notch1 signaling.<sup>103</sup> Similarly, Zhang et al were able to generate large quantities of functional hepatocytes from mouse embryonic stems cells by culturing them in an RCCS bioreactor under  $s\text{-}\mu\text{g}$  in the presence of 20 ng/mL hepatocyte growth factor, 10 ng/mL recombinant human fibroblast growth factor-4, 10  $\mu\text{g}/\text{mL}$  insulin, 5  $\mu\text{g}/\text{mL}$  transferrin, and 5 ng/mL selenium.<sup>104</sup> Interestingly, in human hepatic and biliary tree stem/progenitor cells  $s\text{-}\mu\text{g}$  was shown to promote the generation of 3D spheroids and to stimulate the glycolytic metabolism, but also to favor maintenance of stemness and not the differentiation toward hepatocytes.<sup>105</sup> Further steps toward reconstructing functional hepatic tissue were taken by Wang et al. Embryonic stem cells were seeded onto a biodegradable scaffold and incubated under  $s\text{-}\mu\text{g}$  in a rotating bioreactor.<sup>106</sup> They



differentiated and matured into hepatic-like cells both *in vitro* as well as after a transplantation of these constructs into mice, where they remained viable and functional. Using E15.5 fetal liver cells, containing more hepatic stem/progenitor cells compared to neonatal liver cells, Ishikawa et al obtained functional hepatic tissue including mature hepatocyte and blood vessel-like structures accompanied with bile duct-like structures from  $5\text{-}\mu\text{g}$  culture conditions in an RWV, something they could not achieve using conventional 3D culture techniques.<sup>107</sup> Lastly, spheroids derived from ADSCs cultivated under  $5\text{-}\mu\text{g}$  showed increased proliferative ability, improved multipotency differentiation capacities, and most importantly, more effective potentials to rescue liver failure in a mouse model with carbon tetrachloride-induced acute liver failure than ADSCs derived from constant monolayer culture.<sup>108</sup> Interestingly, these effects seem to be confined to  $5\text{-}\mu\text{g}$ , as experiments with PICM-19 pig liver stem cells cultured for 16 days under  $1\text{-}\mu\text{g}$  during the STS-126 mission revealed only minor differences between flight and ground control samples.<sup>109</sup>

#### 4.6 | General research on embryonic, pluripotent and MSCs

In general, studies on embryonic stem cells exposed to  $\mu\text{g}$  suggested that  $\mu\text{g}$  inhibits the stem cells' differentiation and retains their self-renewal markers.<sup>110</sup> Its inhibitory effect could be due to down-regulation of a number of genes in embryonic stem cells.<sup>46</sup> Similar observations were made by Kawahara et al, who were able to maintain mouse ESCs (mESCs) and embryonic bodies in an undifferentiated state over 7 days by using a 3D CN and leukemia inhibitory factor-free culture media.<sup>111</sup> A 5-day experiment on board the SJ-10 satellite revealed that spaceflight in general had a globally suppressive effect on gene expression in mESCs. The most affected biological processes were involved in the development of the cardiovascular system and in neurodevelopment.<sup>112</sup> During the recent 15-day TZ-1 space mission, it was shown that  $1\text{-}\mu\text{g}$  prevented terminal differentiation of mESCs and promoted cell survival.<sup>113</sup> Studies on murine-induced pluripotent stem cells on the same mission seemed to suggest that  $1\text{-}\mu\text{g}$  promoted the expression of the pluripotency marker Oct4, therefore retaining stemness and resulting in a more dynamic behavior compared with ground controls.<sup>114</sup>

When cultured under  $5\text{-}\mu\text{g}$  in rotary bioreactors in a differentiation medium, mESC-derived embryonic bodies showed a distinct differentiation pattern, which diverged from that observed in mESCs cultivated in spinner flasks using the same medium. While  $5\text{-}\mu\text{g}$  yielded more sca-1<sup>+</sup> progenitors, spinner flasks generated more c-Kit<sup>+</sup> progenitors.<sup>115</sup> Using mESCs, Lei et al have shown that RCCS cultivation allows for a more controlled production of embryonic bodies with more uniform sizes and more regular shapes than those obtained from static culture techniques. They also reported that  $5\text{-}\mu\text{g}$  enhanced mesendoderm differentiation, whereas neuroectodermal differentiation was reduced; an effect the authors suggest was mediated by the Wnt/ $\beta$ -catenin pathway.<sup>116,117</sup>

Human adipose tissue-derived MSCs (ADMSCs) cultured in  $5\text{-}\mu\text{g}$  in standard laboratory incubators alter their proliferation and differentiation. The ADMSCs were exposed for 14 days to an RPM and cultures under two oxygen concentrations: 5% and 20%. The authors showed that gene expression in RPM-exposed cells is differently modulated depending on the oxygen concentration. They also demonstrated that simulated  $\mu\text{g}$  influences the cytoskeleton, whereas oxygen is a key player, influencing the degree of these alterations.<sup>118</sup>

## 5 | CONCLUSION AND FUTURE PERSPECTIVES

Exposing stem cells to  $\mu\text{g}$  conditions increased the current knowledge about tissue engineering of various tissues such as cartilage, bone, vasculature, heart, and liver tissue (Table 1, Figure 2). In addition, stem cells have been investigated in space or with  $\mu\text{g}$  simulators with the purpose to investigate differentiation changes and to study the relevant pathways involved in biological processes which are important for the health problems of space travelers as well as countermeasures for long-term space exploration to Moon and Mars. Tissue engineering in  $\mu\text{g}$  is a new technique to produce organoids, spheroids, or tissues with and without scaffolds and is useful for translational regenerative medicine (Figure 2).

Cancer research in space is currently a novel research field. Cancer research on the ISS comprises studies investigating clinical-grade stem cells for therapeutic use or crystallizing proteins for improved drug discovery and delivery. Other cancer-related projects study 3D cell culturing methods in space and biofabrication of tissues. This novel research should help us to rethink cancer research on Earth with the aim of developing new drugs and cancer treatment strategies.

Only a few studies investigating the impact of simulated  $\mu\text{g}$  on CSCs were published. The results in this field are dependent on cell type and the choice of the  $\mu\text{g}$  simulator. Non-small lung CSCs are rescued from their quiescent state and lose their stemness when subjected to  $\mu\text{g}$  (RPM),<sup>15</sup> whereas human colorectal cancer cell HCT116 exposed to the RCCS showed an elevated but staggered autophagic flux and increased stemness, including CD133/CD44 dual positive cells.<sup>48</sup> CSC generation from different cancer cell types exposed to  $\mu\text{g}$  was successful and  $\mu\text{g}$  simulation will be an excellent method to enrich the CD133-positive CSCs from various cancer cell lines.<sup>55</sup> This relatively small subset of cancer cells exhibits a strong capability of self-renewal, is essentially resistant to chemotherapy, highly metastatic, and believed to be a key factor in tumor survival and progression.<sup>119</sup> Therefore, tests to evaluate the efficacy of chemotherapeutic drugs on cancer cells and CSCs *in vitro* prior to their use in the clinic may be a novel therapy option for cancer patients. The identification of new target structures involved in CSC proliferation and differentiation may furthermore lead to the development of novel tumor-suppressing drugs. Because of differing results obtained when using RPM, RCCS, or HFB devices, more research on different cancer types is necessary to study the biology of cancer stemness.

When comparing results from r- and s- $\mu\text{g}$ , it should not be forgotten that there are some fundamental differences between the two conditions. Cell culture in r- $\mu\text{g}$  in space takes place in an essentially force-free environment without any perturbations of the culture medium except for the naturally occurring diffusion of nutrients and cellular waste products due to local concentration gradients. Cells on an s- $\mu\text{g}$  device such as an RPM, RWV, RCSS, or CN on the other hand experience residual acceleration depending on their distance to the center of rotation, shear forces, and a constant mixture of the cell culture medium.<sup>18</sup> Although these effects are not necessarily detrimental, they introduce additional factors and variations over the course of the cell culture procedure, which will eventually lead to deviations between results from r- and s- $\mu\text{g}$  experiments. The 14-day ESA-SPHEROIDS space mission investigated endothelial cells in space and revealed a scaffold-free formation of spheroids and TS in space.<sup>8,120</sup> Spheroid and TS formation can also be found when endothelial cells were exposed to an RPM. A similar finding was observed during the Shenzhou-8/Simbox space mission (follicular thyroid cancer cells—FTC-133), where spheroid formation was visible postflight. These spheroids were larger as those engineered on the RPM.<sup>121</sup> The postflight analyses of the spaceflight and the RPM samples demonstrated a large number of genes similarly regulated under RPM and spaceflight conditions.<sup>122</sup> Finally,  $\mu\text{g}$  research in space and on Earth is a new technology to support the development of patient-specific therapies and to bring new ideas to the fields of cancer research and regenerative medicine.

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## CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

## AUTHOR CONTRIBUTIONS

D.G.: conception and design, manuscript writing, final approval of manuscript; M.W., T.J.C., P.R., B.P., M.L., J.B.: manuscript writing; M.E., S.K.: administrative support; M.K.: manuscript writing, graphical design.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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