Molecular Genetic Analysis of Neural Stem Cells After Space Flight And Simulated Microgravity on Earth

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ABSTRACT

Understanding how stem cells adapt to space flight conditions is fundamental for human space missions and extraterrestrial settlement. We analyzed gene expression in boundary cap neural crest stem cells (bNCSCs), which are attractive for regenerative medicine by their ability to promote proliferation and survival of co-cultured and co-implanted cells. bNCSCs were launched to space (Space cells), onboard Sounding rocket as free-floating neurospheres or in bioprinted scaffold. For comparison, bNCSCs were placed in random positioning machine to simulate microgravity (Microgravity cells) or cultured under Earth conditions. Using Next-Generation RNA sequencing and data post-processing, a list of genes that were at least two-fold changed between control cells and Space cells were selected for further analysis. Functional clusters of enriched genes were obtained by gene ontology bioinformatics, using the DAVID program, and Ingenuity Pathway Analysis was used to predict functional implications of the identified gene expressions. Space cells upregulated genes related to proliferation and survival, whereas Microgravity cells upregulated genes associated with differentiation and inflammation. Thus, i) space flight provides unique conditions with distinctly different effects on the properties of bNCSCs compared to Earth controls, and ii) may induce post-flight properties that reinforce the utility of bNCSCs for regenerative medicine and tissue engineering.

1 | INTRODUCTION

Humans embarking on space missions are subjected to pronounced physiological stress, mainly from the exposure of the organism to hyper- and microgravity resulting in a slowing of the cardiovascular system, redistribution of body fluids, weakening of the immune system, muscle atrophy, osteopenia, and loss of body mass (Demontis et al, 2017). Space flight conditions have also been shown to alter brain structure and functions with more severe effects after long-term rather than short-term space missions.

Stem cells are fundamental for the development and growth of the organism, for renewal of cell space with a limited lifespan, as well as for tissue regeneration and repair after injury or disease. Stem cells have also emerged as an attractive source of transplants for cell replacement and trophic support, as well as for disease-modifying purposes. Understanding how stem cells adapt to space conditions is thus important for protecting the health of space voyagers (Zhang et al, 2015; Grimm et al, 2020), but also for translational regenerative medicine (Grimm et al, 2018; Imura et al, 2018).

Microgravity is a major physical challenge during space flight and in an extraterrestrial environment. The effect of microgravity has been studied previously on various types of stem cells after space flight, on the international space station, or on earth, using devices designed to simulate microgravity (random positioning machine (RPM) or clinostat). These studies show that microgravity may have beneficial (Chen et al, 2011; Zarrinpour et al, 2017, Baio et al, 2018; Graziano et al, 2018; Camberos et al, 2019; Li et al, 2019), as well as detrimental effects (Yuge et al, 2011; Espinosa-Jeffrey et al, 2013; Blaber et al, 2015; Zhang et al, 2015; Chen et al, 2015, 2016; Mao et al, 2016; Shinde et al, 2016; Acharya et al, 2018; Hatzistergos et al, 2018) on cells.

Although simulated microgravity on earth shares the principles of microgravity in space, stem cells may display distinctly different responses in space compared to microgravity

on earth (Zhang et al, 2015). Therefore, it is crucial to verify findings in "real life", to determine possible undesirable effects not identified in earth experiments, and also to identify possible novel features of potential beneficial translational implications. Here, we explore how space flight conditions, compared to RPM conditions applied on earth, affect gene expression in boundary cap neural crest stem cells (bNCSCs). bNCSCs are a transient neural crest-derived group of cells that self-renew, show multipotency in culture (Maro et al, 2004; Hjerling-Leffler et al, 2005; Aldskogius et al, 2009), and are able to differentiate into neurons and glia *in vitro* and after transplantation to the peripheral (Aquino et al, 2006) or central nervous system (CNS) (Zujovic et al, 2010, 2011; Trolle et al, 2014; Radomska and Topilko, 2017) *in vivo*.

bNCSCs have emerged as a highly attractive resource for regenerative medicine due to their remarkable, beneficial effects on insulin-producing beta-cells (Olerud et al, 2009; Grouwels et al, 2012; Ngamjariyawat et al, 2011, 2012; Lau et al, 2015; Grapensparr et al, 2015), and endangered motor neurons (Aggarwal et al, 2017; Schizas et al, 2018; Leyton-Jones et al, 2020). At the same time, bNCSC-derived astrocytes show extreme resistance to challenges such as oxidative stress (Aggarwal et al, 2017).

bNCSCs were launched into space either as free-floating neurospheres or placed in a bioprinted scaffold. For comparison, bNCSCs were subjected to altered gravity mimicking-space condition, or RPM condition (Microgravity cells), or were cultured without physical challenges in the laboratory. We show striking differences between Space cells and Microgravity cells, with Space cells upregulating genes involved in cell proliferation and survival, whereas Microgravity cells upregulated genes involved in differentiation and inflammation.

2 | MATERIALS AND METHODS

2.1 | Culture of boundary cap neural crest stem cells (bNCSCs)

bNCSCs were isolated in a semi-clonal fashion from transgenic mice harboring red fluorescent protein (RFP) under the universal actin promoter (Vintersten et al, 2004) according to previously published protocols (Hjerling-Leffler et al, 2005; Aldskogius et al, 2009). Briefly, dorsal root ganglia along with boundary caps were mechanically separated from the isolated spinal cord and mechano-enzymatically dissociated using Collagenase/Dispase (1 mg/ml) and DNase (0.5 mg/ml) for 30 mins at room temperature. Cells were plated at 0.5-1×10^5 cells/cm^2 in an N2 medium containing B27 (Gibco) as well as EGF and bFGF (RnD Systems, 20 ng/ml respectively). After 12 h, non-adherent cells were removed together with half of the medium before adding fresh medium. The medium was changed every other day (50% of the medium replaced with fresh medium) until neurospheres could be observed after approximately three weeks of culture. Non-passaged neurospheres between two and three weeks in culture have been frozen and later used for subsequent experiments.

2.2 | Preparation of bioprinted bNCSCs for space flight and earth experiments

bNCSCs were delivered to CELLINK (www.cellink.com) on day three after the start of culture. Spheroid samples were centrifuged and the supernatant discarded. The remaining pellet was mixed in CELLINK Bioink (CELLINK AB, IK1020000303) and printed with crosslinking by immersing the constructs in a 50 mM solution of $CaCl_2$ for two minutes. The printed scaffolds with bNCSC were placed in Eppendorf tubes as three separate droplets.

2.3 | Space flight experiments

bNCSCs in CellINK or as free-floating neurospheres were collected in Eppendorf tubes, which were placed in a Styrofoam box and delivered to the launch site at Esrange-Kiruna (https://www.sscspace.com/ssc-worldwide/esrange-space-center/). The box was placed in the bottom of the rocket MASER 14 (https://www.sscspace.com/maser-14/) one week before

take-off. At the same time, when cells were sent to CELLINK or Esrange, bNCSCs were placed on a bench in the lab in Eppendorf tubes and the medium was not changed during the whole experiment. After the space voyage, the cells were returned to the lab and placed in fresh medium. As an additional control, cells cultured under normal conditions in the incubator were used. The space flight conditions included 6 minutes of microgravity and 6 minutes of hypergravity.

2.4 | Simulated microgravity and altered gravity experiments

In the Institute of Medical Engineering, IMT, Switzerland, an aditional gravity experiment was prepared, which reconstructed the12 min space condition: six min of hypergravity and six min microgravity. Furthermore, a microgravity experiment with 48h simulated microgravity condition was carried out. Both experiments were performed with cells that had been out of the incubator for the same time as those in space (14 days).

2.5 | Post-flight culture, survival and viability assay

On day three after landing the Space cells were delivered to the laboratory and placed in fresh proliferation medium in parallel with Earth controls to grow stem cell mass. Live images were taken daily during the first week of culture, using inverted fluorescence microscopy to identify RFP expressing bNCSCs. The bNCSCs from all conditions were cultured for one to four weeks before they were collected and frozen for gene expression analysis. A separate set of cultures were prepared immediately after landing to test cell survival and viability, using viability labeling protocol based on Calcein AM (live cells; Invitrogen eBioscience, product number 15560597) and Propidium Iodide (dead cells; Sigma-Aldrich, product number 81845-25MG) on Space and Earth control samples.

2.6 | RNA extraction and Next-Generation RNA Sequencing

bNCSC neurospheres derived from Space cells and Earth controls were grown in stem cell medium for one to four weeks after space voyage in the same culture conditions as described

above according to a previously published protocol (Lefler 2005, Aldskogius 2009). RNA was extracted using an RNAEasy Plus Micro kit (Qiagen), according to the manufacturer's instructions. Concentrations were measured using ND-1000 spectrophotometer (NanoDrop Technologies) and subsequently, RNA integrity was controlled by using an Agilent 2100 Bioanalyzer.

Ten ng of RNA was reverse transcribed according to Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit Preparation protocol (ThermoFisher). The cDNA was amplified using Ion AmpliSeq™ Transcriptome Mouse Gene Expression core panel (ThermoFisher) and the primer sequences were then partially digested. Adaptors (Ion P1 Adapter and Ion Xpress™ Barcode Adapter, Life Technologies) were then ligated to the amplicons. Adaptor ligated amplicons were purified using Agencourt® AMPure® XP reagent (Beckman Coulter) and eluted in the amplification mix (Platinum® PCR SuperMix High Fidelity and Library Amplification Primer Mix, ThermoFisher) and amplified. Size-selection and purification were conducted using Agencourt® AMPure® XP reagent (Beckman Coulter). The amplicons were quantified using the Fragment Analyzer^{TM}instrument (Advanced Analytical Technologies, INC.) with DNF-474 High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical Technologies, INC.). Samples were then pooled, eight or less per pool, followed by emulsion PCR on the Ion Chef™ System using the Ion 550™ Kit-Chef (ThermoFisher). The pooled samples were loaded on one Ion 550™ chip and sequenced on the Ion S5XL™ System using the Ion S5 Sequencing chemistry (200 bp read length, ThermoFisher).

2.7 | Data post-processing and analysis

The raw data was converted to the number of reads per transcript using ampliSeqRNA plugin (Illumina, San Diego, USA) for the Torrent Suite Software (Illumina, San Diego, USA). These values were normalized by dividing the read number for each gene for each sample, by the total number of reads for that gene. From these normalized gene expressions, a gene of

interest list was created. First, all genes that were at least two-fold changed between cells outside the incubator and control cells, were removed because these were considered affected by the change in the environment between these groups and could be possible confounders. The remaining genes that were at least two-fold changed between control cells and Space cells were included in the gene of interest list, which was used for subsequent analysis.

DAVID (Huang et al, 2008, 2009) was used to identify functional clusters of enriched genes from the gene of interest list. The DAVID analysis was run using default settings. The same gene of interest list as used for the DAVID analysis was used for Reactome pathway analysis (Fabregat et al, 2018; Jassal et al 2020), which was conducted with default settings.

Ingenuity Pathway Analysis (IPA) was performed by setting Gene only, specific cell selection in Cell-Neurons, Stem Cell, Brain-Cerebral cortex, and CNS cell lines. The data set used was the gene of interest list obtained from the data post-processing step. Comparative analyses were performed on all groups (Space, mimic, Microgravity, normal, outside incubator). Canonical pathways and molecular functions were enriched to cell and stem cell differentiation, adhesion, and proliferation pathways.

3 | RESULTS

3.1 | Space flight cultures show increased survival and proliferation

After their return from the space flight, bNCSCs were placed in proliferation stem cell medium (Fig.1A). In this medium bNCSCs typically grow as neurospheres (Fig.1B), but during the following two days, we detected strongly adhering Space cells, occupying the bottom of the wells (Fig. 1C). On day three, Space cell cultures displayed an exceptional amount of spheres (Fig.1D), which were substantially larger than for bNCSCs cultured on the ground (Fig. 1B). The spheres had to be split 12 times faster in Space cell cultures than in the other culture groups.

bNCSCs, placed in CELLINK Bioink scaffold survived Space conditions compare to the Earth scaffold control cells. Using Calcein to iden tify live cells, and Propidium Iodide to identify dead cells we found a significantly increased number of viable cells in Space cell cultures compared to Earth control ($p < 0.005$); Fig. 2).

3.2 | Space flight and Earth microgravity result in different patterns of gene expression (Table 1).

We created a gene of interest list with those genes that were uniquely, at least two-fold, differentially expressed between Space cell and earth Microgravity cells, by removing genes that showed altered expression as a result of maintaining the cells outside the incubator. We applied this gene of interest list for the Gene ontology analysis using DAVID and identified three functional groups that were highly enriched, namely "Adhesion", "Proliferation" and "Differentiation". We also identified "Immune function" as enriched, however, because we only have neural cells in this culture system, we found the data hard to interpret and they were therefore not considered further. One possible explanation for this enrichment is that some of the other groups, mainly "Adhesion", have a high overlap of their included genes with those associated with immune function. The genes underlying this enrichment from the gene of interest list are shown in Table 1.

For in-depth analysis, we focused on genes with the most prominent alterations in expression, i.e. those that showed at least a two-fold difference in expression levels. Figure 3 presents these genes in the three distinct groups that they represent; Differentiation (A), Proliferation (B), and Adhesion (C). The selected genes are plotted as the Space group compared to the control group of cells, which were kept outside the incubator. All genes which were different between outside incubator control and normal incubator control have been removed from the analysis to eliminate the effect of unnormal, outside incubator

conditions. We therefore only analyzed changes, which could be related to the space conditions.

3.3. | Space flight and RPM exposure resulted in markedly different pathway activation

Genes associated with proliferation, adhesion, and differentiation showed significant differences between Space and Microgravity groups. Reactome Pathway Database revealed interesting differences between simulated Microgravity and Space groups (not shown). Space cells, unlike the RPM group, do not upregulate genes involved in programmed cell death and inflammation (immune system). In contrast, only Space cells show upregulation of genes associated with cell-cell communication and show multiple involvements in different neuronal pathways. These data show that bNCSC subjected to space conditions acquire new properties, which might benefit their survival as well as their potential to support co-cultured, co-printed, and co-transplanted cells

3.4 | Ingenuity Pathway Analysis (IPA)

We next applied the IPA software to the list of all genes which showed at least a twofold change. Here cells are not normalized as compared with the control group. Comparative gene expression ratio between groups such as Space cells Vs Mimic cells, Space cells Vs Outside incubator cells, Outside incubator Vs Normal, Space cells Vs RPM (Microgravity) cells, subjected to IPA software were specifically centered on stem cells type.

After performing top upstream regulator analysis, six genes indicated inhibition of tumor protein TP73, a transcription regulator known to be involved in cellular responses to stress, and in development (Fig. 3A). Further biofunctional analysis of Space cells indicated that the most highly differentially expressed genes affected cellular functions such as differentiation of CNS cells (Fig. 3B). When we focused our analysis on possible effects on cell viability. IPA predicted increased cell viability in Space cells compared to other groups, as evidenced by upregulation of several survival associated genes. Furthermore, genes

regulating additional supportive cellular functions were upregulated, such as differentiation of neuronal precursors, the survival of neural crest cells, as well as migration, activation, and development of embryonic stem cells (Fig. 3C). Genes involved in the differentiation of the CNS cells were SOX10, PTEN, BMP2, and FN1 were predicted upregulated, whereas SHH was predicted downregulated (Fig. 3C).

After performing a comparative analysis of all four groups, the top three affected canonical pathways were the senescence pathway, dopamine signalling, and amyotrophic lateral sclerosis signaling (Fig. 3D). Among all groups in Space cells, the senescence pathway was predicted downregulated, which is supported by genes involved and affected in the pathway (Fig. 3E). Top network analysis was focused on genes involved in cellular development, growth and proliferation, of the nervous system in Space cells. Genes involved showed increased cell viability of embryonic cells in Space cells. Genes which are also involved in the differentiation of neuronal precursors, activation of embryonic stem cells, migration of embryonic cells, and survival of neural crest cells were also upregulated. Comparative analysis of all different groups showed the senescence pathway as one of the top canonical pathways which were indicated as inhibited in Space cells as well as in RPM cells. In conclusion, both RPM cells and Space cells show reduced stem cell senescence, which protects them from the natural cell ageing process, a process that affects the capacity for stem cell renewal.

4 | DISCUSSION

We show that bNCSCs after being exposed to a short period of space flight conditions display a remarkable, and improved viability compared to cells exposed to simulated gravity changes on earth. These space flight-induced cellular changes were associated with significant differences in the activation of pathways for genes involved in proliferation, differentiation,

and cell adhesion, indicating that bNCSCs successfully adapt to the challenging conditions of space flight and even acquire novel properties of benefit for cell function. In a short term perspective, these responses are in line with the impact of physical stress, which makes cells reorganize their cytoskeleton, restructure actin and cadherin complexes and flatten nuclear pores ((Elosegui-Artola et al. 2017), which in turn causes changes in cellular processes such as adhesion, proliferation, and differentiation (Dasgupta and McCollum 2019). The completely different response in simulated microgravity conditions on earth and space flight conditions show that space flight conditions provide a unique environment in affecting bNCSCs.

The Hippo signaling cascade has been associated with cellular response to physical stress exposure (Dasgupta and McCollum 2019) and during a space flight, such stress exposure can be experienced. Subsequent to the physical stress exposure Yes-associated protein 1 (YAP1); one key-player of the Hippo pathway, translocates into the nucleus and affects the expression of target genes (Aragona et al. 2013; Elosegui-Artola et al. 2017). The adaptation to physical stress entails alterations in the expression of genes involved in proliferation and differentiation (Hansen et al, 2015; Meng et al,2016).

During hypergravity, cells experience excessive physical stress. In line with current literature, our results indicate an enhanced proliferation rate by upregulation of signaling cascades like the Hippo pathway, WNT, and GPCR signaling (Aragona et al. 2013) (Fig. 3). The survival and viability of bNCSCs in printed scaffold from Space samples were significantly greater than in Earth control. In contrast, results from previous studies have shown an increased amount of apoptotic cells in hair follicle-derived neural crest stem cell cultures exposed to simulated microgravity compared to 1 *g* conditions (Lin et al, 2016). The improved survival in our experiments may be due to a possible protection of the bNCSCs in

the 3D printed scaffold against the forces exerted during the hypergravity-microgravity conditions.

Our pathway analyses show activation of several pathways that are strongly associated with proliferation, differentiation, and cell adhesion. Pathways that were strongly altered in our space-flown cells compared to earch control groups include VEGF/PDGF and calcium signaling. In addition, the expression of genes related to modifications in cell-extracellular interactions was markedly altered.

VEGF/PDGF signaling is important for the regulation of stem cell properties. Under normal conditions VEGF/PDGF stimulates proliferation and initial differentiation of rat hippocampal neural stem cells *in vitro*, inducing markers for neurons and oligodendroglia (Pelegri et al, 2019). VEGF promotes migration and focal adhesion of human neural stem/progenitor cells derived from olfactory epithelium (Ramírez-Rodríguez et al, 2017), and enhances migration of mesenchymal stem cells (MSCs) by regulating focal cell adhesions, thereby promoting MSC neural differentiation potential (Lyu et al, 2013; Wang et al, 2015).

Previous studies addressing VEGF expression under conditions of altered gravity generally report down-regulation of this growth factor. In simulated microgravity, VEGF was down-regulated in a prostate cancer cell line, and adhesion to substrate reduced, while spheroid formation in the medium was enhanced (Hybel et al, 2020). Simulated microgravity was shown to impair the survival of leukemia cells through a mechanism leading to reduced VEGF signaling (Vincent et al, 2005). Also, space flight as well as in simulated microgravity led to down-regulation of VEGFA and VEGFD in a thyroid cancer cell line (Ma et al, 2014). Thus, the space flight induced activation of the VEGF signaling pathway maybe a critical step to achieve the improved survival and proliferative capacity of neural stem cells in our experiments.

In addition to the activation of the VEGF pathway, we show that the PDGF pathway is also activated. Under normal conditions, PDGF-A induces oligodendroglial progenitor cell (OPC) migration in an ERK-dependent mechanism via regulation of actin reorganization and FAK (Singh et al, 2019). Oligodendrocyte progenitors plated onto a multifunctional film containing PDGF proliferated for at least three days without providing soluble growth factors, while inhibiting the expression of the differentiation marker myelin-basic protein (Moore et al, 2018). In space flight, the PDGF-beta receptor was down-regulated in osteoblasts (Akiyama et al, 1999). Thus, data on the effect of altered gravity conditions on PDGF signaling is extremely limited. Nevertheless, our findings suggest that further analysis of this pathway may be relevant for the regulation of neural stem cell renewal and viability and space conditions.

Calcium signaling pathways were significantly activated in our space flight experiments compared to simulated microgravity controls. Ca^{2+} signaling is important in many cellular processes in neurons, including long term potentiation, intercellular communication, synaptic activity, neuroglial interplay. In general, increased Ca^{2+} is considered beneficial to neuronal activity, providing this increase is moderate and controlled. In recent studies, the number of calbindin immunoreactive neurons in the mouse spinal cord increased after space flight and hindlimb unloading (Porseva et al, 2017; Porseva et al, 2018), whereas labeling for calretinin decreased following hindlimb unloading (Porseva et al, 2018).

Simulated microgravity was also shown to activate PKC, leading to inhibition of calcium signalling in tracheal epithelial cells (Felix et al, 2000). In simulated microgravity, the functions of L-type calcium (LTCC) channels and ryanodine-sensitive Ca^{2+} releases are decreased in vascular smooth muscle (Xue et al, 2011), and LTCC currents are substantially inhibited and calcium channel, voltage-dependent, L type, alpha 1C subunit ($Ca_v1.2$) suppressed at the protein level in an osteoblast-like cell line (Sun et al, 2015). In simulated

microgravity and hypergravity spontaneous calcium oscillations and cytosolic calcium concentration are both increased in a cardiomyocyte cell line (Liu et al, 2020). Increased cytosolic calcium leads to activation of calmodulin-dependent protein kinase II/histone deacetylase 4 (CaMKII/HDAC4) signalling and upregulation of the fetal genes *ANP* and *BNP*, indicating cardiac remodeling (Liu et al, 2020).

Changes in calcium signaling and regulation are closely connected to potassium mediated activities. Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are encoded by the *Kcnma1* gene, ubiquitously expressed in neural, smooth muscle, and neuroendocrine cells, and are implicated in physiological and pathological processes (Szteyn and Singh, 2020). Short- and medium-term microgravity were shown to result in differential activation of cerebral and hindquarter vascular smooth muscle cells (Fu et al 2004). Microgravity-induced BK_{Ca} activation results in increased apoptosis in cerebrovascular smooth muscle cells (Xie et al, 2010). BK_{Ca} controls the synthesis of NO, and changes in this channel are thus likely to influence cardiovascular adaptation to gravity alterations.

Previous studies have shown that cells exposed to gravity alterations modify their interaction with the extracellular environment (Ebnerasuly et al, 2018; Lin et al, 2020). Our gene analyses show that processes involving integrin and collagen synthesis are differently regulated, changes which are in line with what can be expected under the physical stress during space flight.

Relatively few studies that directly compared the effects of space flight with ground-based mimicking experiments have been carried out so far. Previous results mostly indicate that morphological and molecular changes following simulated microgravity and space flight are similar, although often with quantitative differences in the direction of the alterations (Stemenkovic et al, 2010; Talbot et al, 2010; Wuest et al, 2015; Camberos et al, 2019). However, extensive analysis of gene activation in a thyroid cancer cell line subjected to

microgravity in space as well as simulated microgravity, revealed differences in activated pathways under these two experimental conditions (Ma et al, 2014).

Studies on cardiac progenitor cells (Baio et al, 2018), and hematopoeitic stem/progenitor cells have also demonstrated distinct differences in activated pathways (Wang et al, 2019). Furthermore, separate studies, using the same cell type and comparable experimental setups, suggest opposite directions between space flight and simulated microgravity with regard to changes in the expression of adhesion molecules (reviewed in Lin et al, 2020). Space flown bNCSCs displayed a markedly increased proliferation rate, and at the same time an improved viability compared to cells exposed to simulated microgravity. These observations are in line with a previous demonstration that human mesenchymal stem cells (hMSCs) cultured under 10^3 *g* show a significantly increased proliferation rate compared to hMSCs in 1 *g* (Yuge et al, 2006). Along a similar line, adipose-derived stem cells were shown to proliferate at a higher rate when subjected to hypoergravity than 1 *g* conditions (Tavakolinejad et al, 2015).

An important aspect for the comparison of our results with those of existing literature is the temporal dimension. In previous studies, cells were kept in space and thus exposed to microgravity for days or weeks, allowing them to gradually adapt to the microgravity conditions. With the short duration of space flight in our experiments, the period for restoring cellular homeostasis would take place entirely after the return of bNCSCs to 1 *g* conditions on earth. Still, the novel properties of bNCSCs after the flight were maintained for several months, rather than returning to pre-flight properties.

Taken together, we demonstrate that bNCSCs show not only extreme resilience to space flight conditions but actually acquire novel, long-lasting features of benefit for the cells themselves and of potential relevance for translational research and applications. The long-

term maintenance of these properties indicates that short space flight induces epigenetic changes that influence proliferation, differentiation, and adhesion.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no competing interests.

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LEGENDS TO THE FIGURES

Figure 1. Overview of Space and Earth bNCSC cultures at day 0 (A), 2(B,C) and day 3 (D). The cells were cultured in proliferation stem cell medium.

Figure 2. Viability data for surviving cells (Calcein, green) and dead cells (Propidium Iodide, red) in space (left) and on earth (right). Separated bNCSCs, eaqually distributed inside the CELLINK Bioink scaffolds were counted after space flight (left panel) and in Earth controls (right panel). The survival of bNCSCs in Space samples (n=3) was significantly greater ($p<0,01$; Student's t-test) than in Earth samples (n=6) (right graph). A higher proportion of viable cells indicates increased viability and/or increased proliferation capacity of the pace flown cells.

Figure 3. Diagram of activated pathways in bNCSC, subjected to simulated microgravity or after space flight based on Reactome Pathway.

A. Represents genes predicted to be involved in inhibition of top upstream regulator TP73 after performing upstream regulator analysis in space cells.

B. Genes involved in differentiation of central nervous system cell function in space cells.

C. Graphical Representation of genes involved in the viability of embryonic stem cells.

D. Top three canonical pathways represented after performing a comparitive analysis. Groups 1. Space Vs Microgravity; 2. Space Vs Mimic;3. Space Vs Outside incubator; 4. Outside incubator Vs Normal.

E. Heatmap of genes involved in top senescence pathways in different groups 1. Space Vs Microgravity; 2. Space Vs Mimic; 3. Space Vs Outside incubator; 4. Outside incubator Vs Normal.

F. Top network affected in Space cells which are regulated with top 17 focus genes altered involved in cell development, growth and proliferation, nervous system development and function in space, and Microgravity cells.

Table 1. List of analyzed genes and their function(s).

Gene symbol	Gene name	Gene function
ADAM11	ADAM metallopeptidase 11 (disintegrin and metalloprotease)	Cell-cell and cell-matrix adhesion, neurogenesis
CAMK1D	Calcium/calmodulin dependent kinase ID	Activates the CREB-dependent gene transcription, involvement in neural plasticity
CAMKV	CaM kinase vesicle associated	Calmodulin binding, vesicle associated protein
CCL17	C-C motif chemokine ligand 17	T-cell development, activation of mature T-cells in the thymus
COBLL1	Cordon-Blue protein-like 1	Actin-network regulation in neurons
COL16A1	Collagen type XVI alpha chain	Integrin-mediated cell attachment and morphological changes of cell integrity of extracellular matrix
CTSW	Cathepsin W	Regulation of T-cell activity; upregulated by interleukin-2 (IL-2); associated with the inner membrane of the ER of NK- and T-cells
CYB561A3	Cytochrome b561 family member A3	Oxireductase, electron transporter; binds Fe3+ and hemes
DAP	Death associated protein	Interferon-gamma mediated cell death, apoptosis, autophagy
GRB14	Growth Factor Receptor bound protein 14	Interacts with insulin-stimulated tyrosine kinase signaling cascade, inhibitor of MAPK3 phosphorylation
HPCA	Hippocalcin	Calcium binding protein in regulation of voltage-gated Ca^{2+} channels, neuron specificity
HPCAL4	Hippocalcin-like 4	Same as Hippocalcin; possibly involved in regulation of rhodopsin phosphorylation
JAK1/2	Janus Kinase 1/2	Kinase activity in the tyrosine kinase associated signaling cascade; phosphorylates STATs (involvement in IL-2 mediated inflammation)
LGALS4	Galectin 4	Binds lactose, assembly of adherence-junctions; involved in myelination, neurite outgrowth, partially promotes dedifferentiation and proliferation
LY75	Lymphocyte antigen 75	Reduces proliferation of B-lymphocytes antigen presentation in extracellular space in sorting compartment according to similarity of antigens
MOV10	Mov10 RISC complex RNA helicase	A helicase binding RNA; neural development
MUC1	Mucin 1, cell surface associated	Adhesion of epithelial cells (no neuronal function reported)
MYO5B	Myosin VB	Synaptic plasticity, neural shape by transport of organelles along microtubules
MYRF	Myelin regulatory factor	Pre-transcription factor for oligodendrocyte differentiation and myelination
NF1	Neurofibromin 1	Influences GTPase activity of Ras (receptor tyrosine kinase signaling)

NGEF	Neuronal guanine nucleotide exchange factor	Activation of GTPases in the Ras downstream, exchange GDP with GTP (RAC, CDC42 ->reaction to stress)
NOTUM	Notum palmitoleoyl-protein carboxylesterase	Negative key-regulator of Wnt signaling cascade, serine palmitoleoylation for Wnt proteins binding to frizzled receptor
NRIP2	Nuclear receptor interacting protein 2	Downregulation of nuclear receptors such as NR1F2
PARP10	Poly (ADP-ribose) polymerase family member 10	Mono-ADP ribosylation of GSK3B resulting in negative effect on GSK3B kinase activity in Ras downstream
PDGFA	Platelet derived growth factor subunit A	PDGFs are involved in neural processes such as cell survival, ion-channel modulation, neurogenesis, and synaptogenesis;
PKIB	CAMP-dependent protein kinase inhibitor beta	A kinase inhibitor that if overexpressed can lead to several forms of cancer (of prostate mostly); enriched in the cerebellum
RHOU	Ras homolog family member U	Member of the Ras downstream signaling, also mediator in Wnt signaling
SLC17A7	Solute carrier family 17 member 17	Mediates uptake of glutamate into synaptic vesicles
SMAD1/5	SMAD family member 1	Downstream mediators of receptor tyrosine kinase signaling; control of neurogenesis; stimulated by YAP from the Hippo-Pathway
SOS	SOS Ras/Rac guanine nucleotide exchange factor	Promoting the exchange of GDP with GTP on the GTPase Ras
SOX3	SRY-box 3	Transcription factor preventing neural development
STAT6	Signal transducer and activator of transcription 6	Involved in JAK/STAT signaling cascade induced by e.g. IL-2, promoting inflammation and apoptosis; enhanced expression in ALS
TMEM176B	Transmembrane protein 176B	Maturation of dendritic cells, development of cerebellar granule cells; contribution to CD8+ T-cell tumor growth inhibition lack of TMEM176B leads to enhanced antitumor activity
WNT3	WNT family 3	Ligand for frizzled receptor in the Wnt signalling cascade; involved in embryogenesis: mesoderm and primitive streak formation; regeneration of dorsal root ganglion cell axons
ZBTB16	Zinc finger and BTB domain	Mediator in poly-ubiquitination by E3- ubiquitin protein ligase; transcriptional repressor of genes involved in cell-cycle progression