SPRINGER BRIEFS IN SPACE LIFE SCIENCES

Günter Ruyters
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Biotechnology in Space

SpringerBriefs in Space Life Sciences

The extraordinary conditions of space, especially microgravity, are utilized for research in various disciplines of space life sciences. This research that should unravel – above all – the role of gravity for the origin, evolution, and future of life as well as for the development and orientation of organisms up to humans, has only become possible with the advent of (human) spaceflight some 50 years ago. Today, the focus in space life sciences is 1) on the acquisition of knowledge that leads to answers to fundamental scientific questions in gravitational and astrobiology, human physiology and operational medicine as well as 2) on generating applications based upon the results of space experiments and new developments e.g. in non-invasive medical diagnostics for the benefit of humans on Earth. The idea behind this series is to reach not only space experts, but also and above all scientists from various biological, biotechnological and medical fields, who can make use of the results found in space for their own research. SpringerBriefs in Space Life Sciences addresses professors, students and undergraduates in biology, biotechnology and human physiology, medical doctors, and laymen interested in space research. The Series is initiated and supervised by Dr. Günter Ruyters and Dr. Markus Braun from the German Aerospace Center (DLR). Since the German Space Life Sciences Program celebrated its 40th anniversary in 2012, it seemed an appropriate time to start summarizing – with the help of scientific experts from the various areas - the achievements of the program from the point of view of the German Aerospace Center (DLR) especially in its role as German Space Administration that defines and implements the space activities on behalf of the German government.

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Foreword

"Bio(techno)logy and gravity—a strange pair of terms at first glance!" With this rather surprising statement the authors of this latest booklet *Biotechnology in Space* in the series SpringerBriefs in Space Life Sciences introduce their topic. Not unexpectedly, the authors demonstrate in seven chapters following the introduction impressively that there is indeed a strong relationship between these terms: in fact, gravity has not only influenced the origin, distribution, and the evolution of life in general; also changes in gravity, especially the lack of gravity, i.e., the microgravity conditions of spaceflight, exert a marked influence on bio(techno)logical processes.

In the introductory chapter the authors describe the programmatic background and some of the early biotechnological research topics in the respective space programs of Germany and worldwide. As theoretical considerations had promised, processes such as free-flow electrophoresis, electro-cell fusion, and protein crystallization are all improved in microgravity as was shown by space experiments already in the 1970s and 1980s.

Chapters 2–4 deal in detail with the topic of protein crystallization in space. After providing some information on the theoretical background, early successes in structure elucidation by microgravity experimentation are given in Chap. 2. Chapter 3 focuses on experiments performed more recently on the International Space Station (ISS), while Chap. 4 describes the advantages of space experimentation in the context of drug discovery and drug design. The authors describe striking examples for the progress in structure determination in this rather application-oriented field of research, the results sometimes even leading to the foundation of pharmaceutical start-up companies.

In Chaps. 5–7 the focus is switched to cell biology and the role of gravity in cellular processes and cell functions. Chapter 5 provides an introduction into the topic and describes the role of gravity in several mostly human cell types. Recent findings of space experiments and accompanying ground research finally led to a hypothesis how gravity is perceived by these cells.

In Chap. 6 the authors concentrate on the more applied aspects of cell-biology research in space, namely, on tissue engineering in microgravity. Results of recent space research on cartilage, bone, endothelial cells, and thyroid cancer cells are

summarized showing that microgravity stimulates the formation of three-dimensional spheroids or tubular-like structures. The knowledge obtained from these space experiments leads to a better understanding of such process on Earth with great potential for application in the area of cell aggregate formation and pharmaceutical drug testing.

In Chap. 7 the contribution of space research in the context of cancer research is highlighted. Thyroid, breast, and skin cancer research under space conditions is described. The results obtained can be used to rethink cancer research with the aim of developing new drugs or improving cancer research strategies on Earth.

The booklet closes with an outlook on the future potential of bio(techno)logy research in space. The perspectives for future success stories in the area of protein crystallization as well as in cell biology are certainly there, especially with the further scientific utilization of the ISS in an international framework of coordination and cooperation.

Bonn, Germany
October 2017

Günter Ruyters
Markus Braun

Preface to the Series

The extraordinary conditions in space, especially microgravity, are utilized today not only for research in the physical and materials sciences—they especially provide a unique tool for research in various areas of the life sciences. The major goal of this research is to uncover the role of gravity with regard to the origin, evolution, and future of life, and to the development and orientation of organisms from single cells and protists up to humans. This research only became possible with the advent of manned spaceflight some 50 years ago. With the first experiment having been conducted onboard Apollo 16, the German Space Life Sciences Program celebrated its 40th anniversary in 2012—a fitting occasion for Springer and the DLR (German Aerospace Center) to take stock of the space life sciences achievements made so far.

The DLR is the Federal Republic of Germany's National Aeronautics and Space Research Center. Its extensive research and development activities in aeronautics, space, energy, transport, and security are integrated into national and international cooperative ventures. In addition to its own research, as Germany's space agency the DLR has been charged by the federal government with the task of planning and implementing the German space program. Within the current space program, approved by the German government in November 2010, the overall goal for the life sciences section is to gain scientific knowledge and to reveal new application potentials by means of research under space conditions, especially by utilizing the microgravity environment of the International Space Station (ISS).

With regard to the program's implementation, the DLR Space Administration provides the infrastructure and flight opportunities required, contracts the German space industry for the development of innovative research facilities, and provides the necessary research funding for the scientific teams at universities and other research institutes. While so-called small flight opportunities like the drop tower in Bremen, sounding rockets, and parabolic airplane flights are made available within the national program, research on the ISS is implemented in the framework of Germany's participation in the ESA Microgravity Program or through bilateral cooperations with other space agencies. Free flyers such as BION or FOTON satellites are used in cooperation with Russia. The recently started utilization of Chinese spacecrafts like Shenzhou has further expanded Germany's spectrum of flight

opportunities, and discussions about future cooperation on the planned Chinese Space Station are currently underway.

From the very beginning in the 1970s, Germany has been the driving force for human spaceflight as well as for related research in the life and physical sciences in Europe. It was Germany that initiated the development of Spacelab as the European contribution to the American Space Shuttle System, complemented by setting up a sound national program. And today Germany continues to be the major European contributor to the ESA programs for the ISS and its scientific utilization.

For our series, we have approached leading scientists first and foremost in Germany, but also—since science and research are international and cooperative endeavors—in other countries to provide us with their views and their summaries of the accomplishments in the various fields of space life sciences research. By presenting the current SpringerBriefs on muscle and bone physiology we start the series with an area that is currently attracting much attention—due in no small part to health problems such as muscle atrophy and osteoporosis in our modern aging society. Overall, it is interesting to note that the psycho-physiological changes that astronauts experience during their spaceflights closely resemble those of aging people on Earth but progress at a much faster rate. Circulatory and vestibular disorders set in immediately, muscles and bones degenerate within weeks or months, and even the immune system is impaired. Thus, the aging process as well as certain diseases can be studied at an accelerated pace, yielding valuable insights for the benefit of people on Earth as well. Luckily for the astronauts: these problems slowly disappear after their return to Earth, so that their recovery processes can also be investigated, yielding additional valuable information.

Booklets on nutrition and metabolism, on the immune system, on vestibular and neuroscience, on the cardiovascular and respiratory system, and on psycho-physiological human performance will follow. This separation of human physiology and space medicine into the various research areas follows a classical division. It will certainly become evident, however, that space medicine research pursues a highly integrative approach, offering an example that should also be followed in terrestrial research. The series will eventually be rounded out by booklets on gravitational and radiation biology.

We are convinced that this series, starting with its first booklet on muscle and bone physiology in space, will find interested readers and will contribute to the goal of convincing the general public that research in space, especially in the life sciences, has been and will continue to be of concrete benefit to people on Earth.

Bonn, Germany — Günter Ruyters
Bonn, Germany — Markus Braun
July 2014

DLR Space Administration in Bonn-Oberkassel (DLR)

The International Space Station (ISS); photo taken by an astronaut from the space shuttle Discovery, March 7, 2011 (NASA)

Extravehicular activity (EVA) of the German ESA astronaut Hans Schlegel working on the European Columbus lab of ISS, February 13, 2008 (NASA)

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Chapter 1
Biotechnology, Cell Biology and Microgravity

Günter Ruyters, Christian Betzel, and Daniela Grimm

Abstract Gravity, a physical factor being available and constant on Earth since its origin some three and a half billion years ago, has governed the origin, the distribution and evolution of life and still does so. To fully understand its importance, experiments under space conditions, i.e. in the absence of gravity, have been performed since the advent of spaceflight in the middle of the last century. Projects in biotechnology and cell biology are in the focus of this booklet. Due to the lack of gravity, i.e. lack of sedimentation, thermal convection, and—in fluids—hydrostatic pressure, processes such as free-flow electrophoresis and electro cell fusion have been shown to lead to increased separation or fusion in microgravity, respectively. Improved crystallization of biological macromolecules has enabled progress in structure determination with positive consequences, for instance, in drug discovery and design. Cell biological research in space provided a better understanding of the physiological functioning of cells and organisms and led to applications in tissue engineering such as the growth of bone, cartilage and artificial vessels. Also, cancer research has benefitted from cell research in microgravity.

Keywords Free-Flow Electrophoresis • Electro Cell Fusion • Protein Crystallization • Cell Biology • Microgravity Conditions

1.1 Introduction

Bio(techno)logy and gravity—a strange pair of terms at first glance! Before we try to demonstrate the context of these terms, we will start with the necessary definition and a look into history. The term "biotechnology" is composed of the three Greek words "bios" (life), "techné" (art, capability) and "logos" (rationale principle, science). A respective definition by Rehm and Präve (1994) modified by Gassen et al. (1999) consequently states: "Biotechnology is the science and knowledge about the utilization of biological systems (organisms as well as biological processes) in the frame of technical processes and industrial production". In its brochure "Biotechnology 2000", the German Ministry of Research and Technology keeps it simpler by saying: "Biotechnology is the

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technical application of biology" (BMFT 1991; BMBF 2010). The definition that is most widely used today is the one developed by the OECD: "Biotechnology is defined as the application of science and technology to living organisms as well as parts, products or models thereof, to alter living or non-living material for the production of knowledge, goods and services" (OECD 2001).

Irrespective of the question which definition the dear reader may prefer—the use of biological processes or organisms probably began some 8000 years ago with the production of food. Since then several eras of biotechnology with characteristic events or products can be distinguished as follows (Table 1.1) (BMFT 1991):

Table 1.1 Simplified timely development of biotechnology (modified after "Biotechnology 2000", edited by the German Ministry for Research and Technology, 1991)

Era	Time	Examples for processes or products
Pre-scientific Biotechnology (Pre-Pasteur-Era)	ca. 6000 BC until 1865	Production of food (e.g. beer in Babylon, wine and vinegar in Assyria, soy sauce in China, sourdough in Egypt)
Microbial Biotechnology (Pasteur-Era)	1865–1940	Proof of various fermentation processes, citric acid production by fungi, plant and animal cell culture, penicillin production by fungi
Classical Biotechnology (Era of Antibiotics)	1940–1975	Viral vaccines and antibiotics, use of enzymes in detergents, biogas, industrial alcohol, waste water purification
Modern (New) Biotechnology	Since 1975	Diagnostics based on monoclonal antibodies, therapeutical human proteins, genetically modified plants, clone production of organisms (e.g. sheep "Dolly")

The question though is: What is all this to do with gravity? Gravity is ubiquitous on Earth. From simple physical or chemical to complex biological systems—it influences everything that happens on our Earth. Moreover, gravity and life have been inseparably linked in the evolution on our planet for around three and a half billion years. Therefore, it is not surprising that gravity with its physical consequences such as sedimentation, thermal convection, and—in fluids—hydrostatic pressure should then play an important role not only for whole organisms, but also when it comes to biological and biotechnological processes. Frequently, the role of gravity is more than obvious: objects fall to the ground, water flows downhill, and gas bubbles rise up in boiling water. In other natural and technical processes, however, gravity's influence is not immediately apparent, so that its significance may only be uncovered through experiments in the absence of gravity, i.e. in weightlessness or under microgravity conditions.

To be more precise: In physical terms, an object is weightless when it is in free fall. A ball thrown into the air is similarly in a state of free fall, meaning that it is weightless. It is flying along a so-called ballistic trajectory. Generally, all states of weightlessness represent forms of free fall. However, free fall is an ideal condition that is almost never found in reality. All falling bodies are exposed to spurious accelerations of different intensity caused by, for instance, air drag and natural vibrations.

This is why, instead of weightlessness, the term ‘microgravity’ has come to be used to describe extremely low gravity present, for instance, onboard of spacecrafts.

In order to elucidate the role of a certain physical factor scientists usually change the magnitude or direction of this factor or switch it off completely. For gravity, this became possible only with the advent of spaceflight in the late sixties of the last century. At first, opportunities for space research were limited to the Soviet Union and the United States. During various Sputnik and Discovery flights animals, plants and microorganisms were brought into microgravity conditions and analyzed for changes. Manned missions were to follow. After Jurij Gagarin’s and John Glenn’s maiden flights in 1961 and 1962, respectively, the Soviets developed their Salyut space stations, reentry satellites and later the MIR space station. The US established their space programs Mercury, Gemini, Apollo, and space station Skylab. From the research point of view, however, it is fair to say that emphasis in those days was on the control and maintenance of health and performance of astronauts and cosmonauts rather than on curiosity driven life sciences research about the influence of gravity changes on organisms.

The European countries including Germany had no possibilities for space research in those years except some scarce cooperative efforts between scientists. The situation changed, when the US started the development of the Space Shuttle System and looked for cooperation partners. In 1973, the European countries decided to participate in the Space Shuttle System by contributing the Spacelab for research under microgravity conditions. This decision also initiated space research programs especially in materials and life sciences in various countries; in particular in Germany, that had the leadership in the development of Spacelab, the programmatic, organizational and financial basis was laid. As preparation for the scientific utilization of Spacelab also the TEXUS sounding rocket program was established with its first flight in 1977 providing 6 min of microgravity. With Spacelab 1 the Spacelab flights and their scientific utilization successfully began in 1973. Later the Bremen Drop Tower, parabolic flights with airplanes and the utilization of various reentry satellites complemented the suit of flight opportunities, then available for German scientists to perform what is loosely named “microgravity research”.

1.2 Programmatic Background and Early Research Topics

In preparation of the above mentioned scientific utilization of the American Space Shuttle equipped with the European Spacelab experts gathered in Europe and especially in Germany in the 1980s to define the goals, themes, and activities of this joined endeavor. In the frame of the foundation of the German Space Agency (DARA = Deutsche Agentur für Raumfahrtangelegenheiten) in 1989 that was later incorporated into the German Aerospace Center DLR, the space program was updated. Especially for life sciences, three program papers were written, namely on biotechnology, biology and space medicine.

Some of the topics selected for space research in biotechnology were:

- Biochemistry and biophysics of biotechnologically relevant cells
- Cell culturing and bioreactor technology
- Hybridoma formation by cell fusion
- Biotechnological separation techniques such as free-flow electrophoresis
- Crystallization of organic macromolecules, hereinafter referred to as protein crystallization

Later, the restriction on biotechnologically relevant cells was judged as not meaningful, since gravity significantly influences all cells. So, cell biology in general became an important topic of biological space research (see Chaps. 5–7) as well as the investigation of the role of gravity on the development and behavior on plants and animals.

In the following, some of the early space projects in biotechnology are briefly summarized. The idea behind all these projects is the theoretical consideration that microgravity, i.e. the lack of sedimentation, thermal convection, and hydrostatic pressure should be beneficial for biotechnological separation or fusion techniques as well as for crystal growth of biological macromolecules.

1.2.1 Free-Flow Electrophoresis

Free-flow electrophoresis (FFE), also known as carrier-free electrophoresis, is a matrix-free electrophoretic separation technique. FFE is an analogous technique to capillary electrophoresis with a comparable resolution that can be used for scientific questions, were semi-preparative and preparative amounts of samples are needed. It is used to quantitatively separate samples according to differences in charge or isoelectric point. Because of the versatility of the technique, a wide range of protocols for the separation of samples like rare metal ions, protein isoforms, multiprotein complexes, peptides, organelles, cells, DNA origami, blood serum and nanoparticles exist. The advantage of FFE is the fast and gentle separation of samples dissolved in a liquid solvent without any need of a matrix, like polyacrylamide in gel electrophoresis. This ensures a very high recovery rate since analytes do not adhere to any carrier or matrix structure. Because of its continuous nature and high volume throughput, this technique allows a fast separation of preparative amounts of samples with a very high resolution. Furthermore, the separations can be conducted under native or denaturing conditions. FFE was developed in the 1960s by Kurt Hannig at the Max-Planck-Institute for Biochemistry in Martinsried, Germany (Hannig and Heidrich 1990). Until the 1980s, it was a standardized technology for the separation of cells and organelles.

FFE was even tested in space to minimize the sedimentation under microgravity. Based on theoretical considerations described above, scientists expected an improvement of separation in microgravity (Friedrich et al. 1994). In fact, as shown in Fig. 1.1 for erythrocytes of different organisms, these expectations were met in space experiments such as on ASTP (Apollo-Soyuz-Test-Project) in 1975—an early

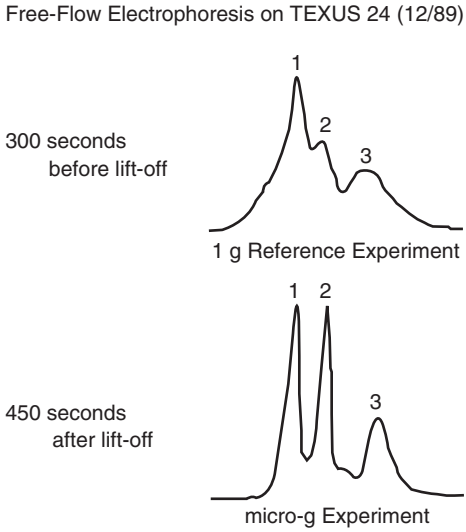

Fig. 1.1 Electrophoretic separation of erythrocytes from rat (*1*), guinea pig (*2*), and rabbit (*3*) during the flight of TEXUS 24 in December 1989 (modified after Hannig et al. 1990)

cooperation between the US and the former Soviet Union—as well as on TEXUS sounding rocket flights.

1.2.2 Electro Cell Fusion

Similar considerations led to cell fusion experiments. With this method, cells with different properties are forced by an electric pulse to fuse, thereby leading to hybrid cells with combined or even new properties. The goal behind was to create cells with properties that would lead to biotechnological applications. Also here, the theoretical expectations were met: In a number of TEXUS sounding rocket flights between 1985 and 1990 as well as during the German Spacelab mission D-2 in 1993 human cells or plant protoplasts were subjected to electro cell fusion. The yield was increased by a factor of up to ten compared to the respective ground controls in some space experiments. Figure 1.2 shows as an example the successful fusion of tobacco protoplasts with different properties, here with and without vacuoles.

After the successful confirmation of the theoretical considerations, however, further experimentation concerning FFE and cell fusion was stopped in the German space program due to the scarce flight opportunities and the high costs of space experiments making a routine utilization of microgravity less probable. Nevertheless, US and Japanese scientists continued with successful experiments in the Space Shuttle era in the 1980s and 1990s in the frame of the NASA and JAXA space programs, respectively (e.g. Kobayashi et al. 1996). Interestingly enough, new emerging space countries such as India or China recently repeated similar experiments during the first phases of their respective research programs

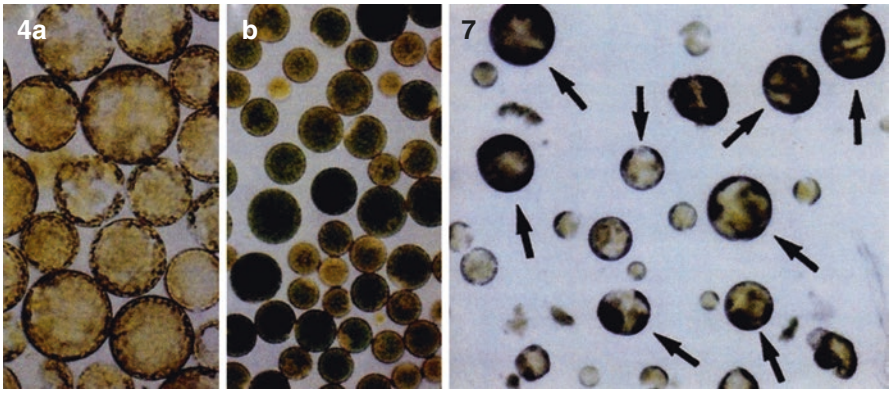

Fig. 1.2 Fusion of tobacco protoplasts with (4a) and without (b) vacuoles to hybrid cells (7) in microgravity during the flight of TEXUS 17 in 1988 (modified after Mehrle et al. 1989)

in order to get accustomed to the special conditions and boundaries of space experimentation.

However, progress in ground experimentation was achieved making space experimentation less attractive. Nevertheless, the methods as such remain still promising: Recent reviews emphasize e.g. the importance of cell electrofusion for new applications such as for antibody and cancer vaccine production (Kanduser and Usaj 2014) or new developments in FFE by miniaturization and application of microfluidic devices (Kohlheyer et al. 2008).

1.2.3 Protein Crystallization in Space

The idea to use microgravity to improve the crystallization of organic macromolecules was, in fact, again born in Germany. Its pioneer was Professor Walter Littke (Freiburg University) who, in 1981, carried out his first experiment during the TEXUS-3 rocket flight. And indeed, in a short spell of microgravity lasting barely 6 min he managed to produce 100 µm long crystals of the enzyme beta-galactosidase. Further experiments on TEXUS and, a little later, on the first Spacelab mission in the year 1983 produced encouraging results, i.e. beta-galactosidase crystals 27 times larger than those produced on Earth, and crystals of lysozyme that were larger by a factor of 1000 (Littke and John 1984). Crystallization under microgravity appeared to be the method of choice. But setbacks were inevitable. A series of technical failures and unfortunate mistakes made in the run-up to the experiments soon gave rise to—sometimes harsh—criticism in the science community.

In addition to hardware developed in various space programs worldwide it was in particular the APCF (Advanced Protein Crystallization Facility) and the later

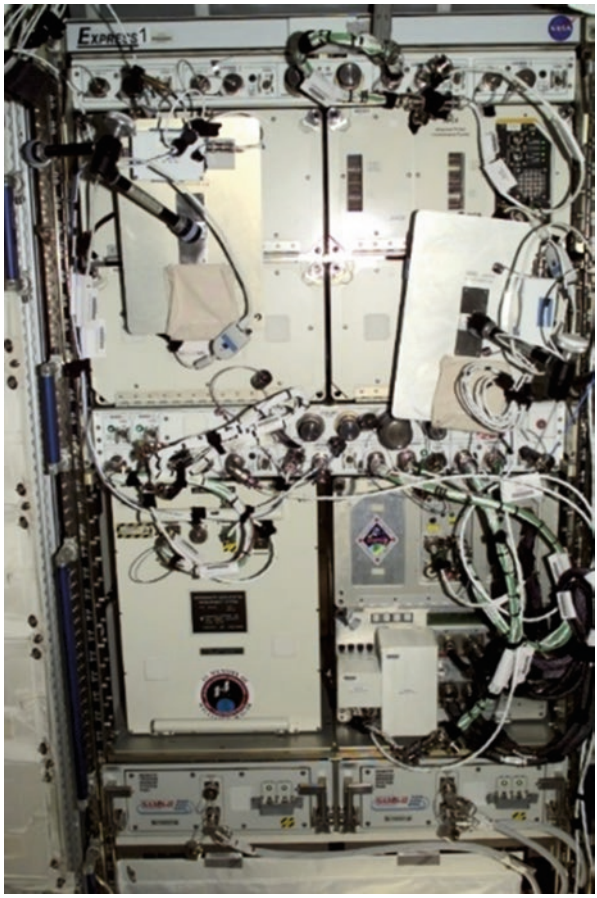

Fig. 1.3 The European APCF (Advanced Protein Crystallization Facility) onboard of ISS (Photo NASA)

constructed enhanced version of APCF with its diagnostic tools that managed to produce the desired results more frequently since the early nineties (Figs. 1.3 and 1.4). Developed by the German industry and successfully used by ESA on shuttle missions and later on the International Space Station (ISS), APCF supported German scientists in their attempts to significantly improve their understanding of the structures of a variety of molecules, some of which they managed to crystallize for the first time.

About 86 space-borne projects involving protein crystallization were carried out as part of the German space program between 1981 and the end of 2016, from COSIMA satellite flights (1988–1991) to US shuttle missions (1992–2003) to experiments on the ISS. Receiving funds from the DLR Space Agency or its predecessor organizations, these projects brought together about 20 teams of scientists from different universities and Max-Planck-Institutes in Germany. Also, US and

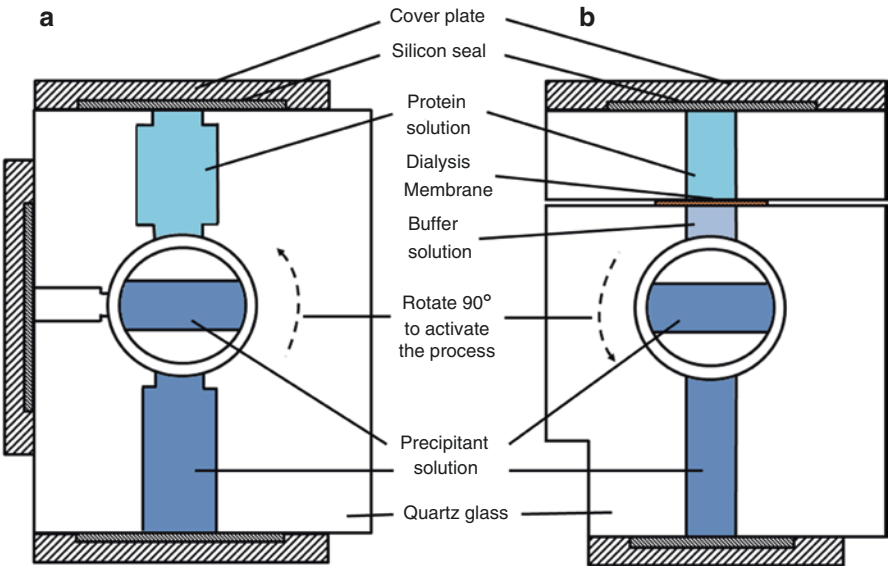

Fig. 1.4 Two types of APCF (Advanced Protein Crystallization Facility) reactors. (**a**) Free interface diffusion reactor. (**b**) Dialysis reactor

Japanese scientists funded by their space agencies NASA and JAXA, respectively, were and still are heavily involved in protein crystallization projects in space. In the next chapters, some of the successes especially of the German space program will be summarized.

1.2.4 Cell Biology in Space

First cell biology experiments in space were performed already in the 1970s. However, the focus was not so much on application of the results achieved in biotechnology, but rather on understanding the role of gravity on cell proliferation, growth and physiological processes. One of the most convincing early results was the finding of Cogoli and his coworkers in experiments during the first Spacelab mission that proliferation of lymphocytes was severely inhibited in microgravity (Cogoli et al. 1984; Cogoli and Tschopp 1985).

To a certain extent, this finding and the results of follow-up experiments laid the foundation of immune system research in space, which is even today one of the cornerstones not only of the German Space Life Sciences Program. Chapter 5 will, therefore, deal in detail with this topic and will also summarize the role of gravity for bone, cartilage, endothelial and cancer cells.

In addition, several more recent experiments have demonstrated that microgravity induces a three-dimensional growth behavior in different cell types

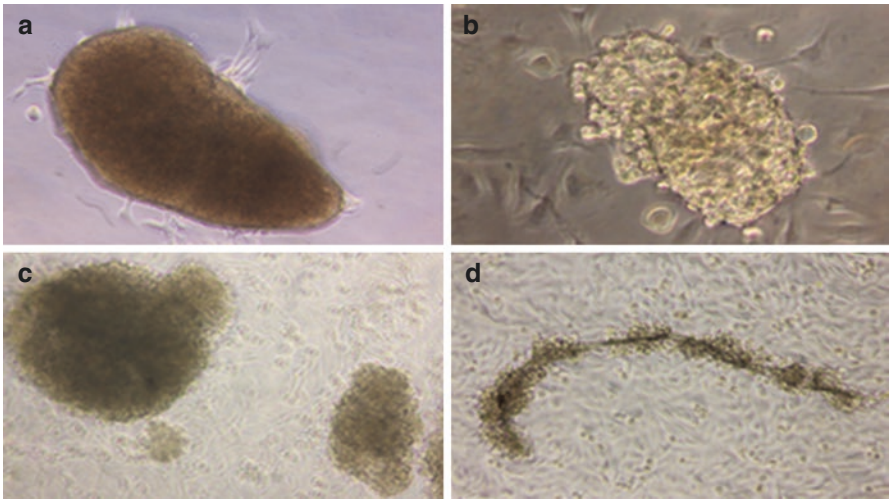

Fig. 1.5 (**a–c**) Multicellular spheroids of different human cell types exposed to the random positioning machine (RPM). (**d**) Endothelial cells form a tubular structure and also grow as a two-dimensional monolayer after RPM exposure

closely representing the *in vivo* situation in the human body offering unique conditions to facilitate scaffold-free development of multicellular aggregates or even organotypic tissue (Fig. 1.5; see Chap. 6; Aleshcheva et al. 2016; Grimm et al. 2014). Finally, cancer research in space has provided impressing evidence for microgravity-induced changes in physiology and structure of cells leading to new approaches in cancer treatment strategies (Becker and Souza 2013; see Chap. 7).

1.3 Perspectives

Biotechnological research in space today is focusing mainly on two areas, namely on protein crystallization in order to achieve progress in structure determination and on cell biology for understanding the role of gravity for proliferation, growth and physiological functioning of cells and organisms. In protein crystallization, especially projects with the goal to obtain more and deeper insights about the crystallization process itself have got much attention and are in the focus of latest research activities, especially on the ISS. Also, application of structural data obtained from space-grown crystals supporting drug discovery and drug design have become very important. Similar goals are valid for cell biology in space: Scientists try to better understand the role of gravity especially in cells that are interesting with respect to growth of vessels, cartilage or bone or help to clarify causes and details of certain diseases such as cancer. Promising results have already been achieved as will be shown in the next

chapters. With the International Space Station being used until at least 2024 further success stories are to be expected.

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Chapter 2
Protein Crystallization in Space: Early Successes and Drawbacks in the German Space Life Sciences Program

Günter Ruyters and Christian Betzel

Abstract The utilization of microgravity for improving protein crystallization and thereby structure determination started in the early 1980s onboard of TEXUS sounding rockets and of the US Space Shuttle. After the successful pioneering work by Prof. Littke, especially the German space life sciences program put much effort into this topic. In spite of some technical and methodological drawbacks, early successes could be obtained as well. In some cases, microgravity experiments enabled crystallization of certain molecules for the first time; in other examples, improved crystals led to a better structure determination with important application potential for structure-function-analysis or even for drug design. Especially after the development of the APCF (Advanced Protein Crystallization Facility) by German industry on contract by ESA and its utilization in Spacelab missions and on the International Space Station ISS, the potential of microgravity for the improvement of crystallization and structure elucidation became clearly visible.

Keywords Protein Crystallization • Microgravity Conditions • Space Hardware Structure Elucidation • Archaea Surface Proteins • Misletoe Lectin • RNA Molecules

2.1 Introduction: Nobel Prize for Clarification of Ribosome Structure

As pointed out in the introductory chapter, the idea to utilize microgravity for improving protein crystallization was born in Germany with the first space experiments being performed by Littke and co-workers onboard of TEXUS sounding rocket flights in 1981 and during the first Spacelab mission in 1983 (Littke and John 1984). Possibly, within the 1970s and 1980s some crystallization experiments were performed by Russian and Chinese scientists, however only incomplete records of these experiments exist.

Nearly 30 years later, in October 2009 namely, the Nobel Prize Committee awarded the Nobel Prize in Chemistry to two scientists working in the USA, Venkatraman Ramakrishnan and Thomas A. Steitz, and to Israel's Ada E. Yonath for their ground-breaking work on the structure and function of ribosomes.

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From 1979 until 1983 Ada E. Yonath was a guest professor at Berlin's Max Planck Institute for Molecular Genetics; from 1986 until 2004 she headed a Max Planck research group at the German Electron Synchroton (DESY) in Hamburg. During this period she was involved with her experiments in a series of space missions, utilizing conditions of weightlessness to improve ribosome crystallization. By the way, for the sake of simplicity we will use the term protein crystallization consistently to cover other organic macromolecules as well, such as nucleic acids, or protein and nucleic acid complexes such as ribosomes.

It is with the help of ribosomes that organisms produce their necessary protein molecules based on the DNA information. This, in a manner of speaking, makes ribosomes the factories of life. To understand their function one has to know their three-dimensional structure at high resolution. Considering that protein structures were already published and deposited more routinely in the late 1970s, for ribosomes structure it took until the year 2000—although the first few discoveries had appeared also at that time, when Yonath began to produce ribosome crystals and explore their structure with the help of X-rays.

Early attempts had produced extremely thin and fragile ribosome crystals that were unsuitable for X-ray diffraction analysis. At this time the option of research in microgravity conditions became available. The theory in the late seventies had been that, given the absence of sedimentation (particles depositing at the bottom of a fluid) and gravity-driven convection (transfer of thermal energy by means of particle transport) in a weightlessness condition, the crystals that would form were likely to be larger and of greater purity. Although as we know today crystal growth is affected by a number of other factors as well, the assumption, on principle, had been correct and has been followed by many scientists ever since. These considerations that led Yonath in the late eighties to develop an interest in experiments under microgravity.

In the period from 1988 until 1995 Yonath sent her experiments on more than twelve space missions. By doing so, she attempted to improve ribosome crystallization on COSIMA satellite missions (Erdmann et al. 1989) and several American Space Shuttle flights including Germany's Spacelab mission D-2 in 1993 with the German astronauts Ullrich Walter and Hans Schlegel onboard. This was when first signs of progress became apparent. The crystals grown during Shuttle/Spacelab missions D-2 (1993), IML-2 (1994) and USML-2 (1995) were larger, rounder and more evenly shaped, thus pointing and guiding the way to further experiments on Earth, which then ultimately ended up in a successful elucidation of ribosome structures, and finally the Nobel Prize (van Noorden 2009).

As will be now shown, Nobel laureate Ada Yonath's crystallization of ribosomes is not the only achievement that deserves attention. Five further particularly successful examples will be presented in more detail. Among them are results of two scientific teams within DLR's microgravity research program that—interesting enough—cooperated closely with Prof. Yonath: Christian Betzel from the University Hamburg at DESY and Volker Erdmann from Berlin Free University, winner of the Leibniz Prize and long-standing spokesman of a collaborative research centre (SFB) at the German Research Foundation (DFG). But before doing that, a few theoretical considerations will provide some further background to the topic in general.

2.2 Some Thoughts on the Theoretical and Methodological Background

Within the last 20 years methods, techniques, instrumentation, software and X-ray radiation sources applied in X-ray crystallography were continuously and substantially further developed and improved, as protein crystallography till now is the most important method and working horse to analyze biomolecules, protein or nucleic acids, at atomic resolution (Su et al. 2015; Lattman and Loll 2008). Only structure function analysis at high and close to atomic resolution provides essential information towards obtaining insights about the various, complex and for each bio-macromolecule unique function. This fundamental knowledge provides for example information required to support the development and design of new pharmaceuticals to treat chronic or infectious diseases, or is providing the required information supporting to engineer enzymes for particular biotechnological applications. However, the use of X-ray crystallography to determine protein structure requires the production of well-ordered protein crystals of sufficient quality and size, named or entitled X-ray suitable crystals. Even today, after installation of third generation and high brilliant synchrotron radiation sources and upcoming X-ray Free Electron lasers (XFELs), which require only nano- or micro-sized crystals (Martin-Garcia et al. 2016), the overall demand to produce well-ordered high quality crystals remains, which means crystals with low internal mosaicity and low incorporation of impurities. The growth of such diffraction quality crystals of biomolecules is often difficult and depends on the molecule itself. For example, high flexibility of a protein or subdomains of the protein, often connected to the function of the biomolecule, is known to restrict crystal growth (Giegé 2013). And till now protein crystal production is the well-known bottleneck of the method and the time limiting factor of a crystal structure analysis (McPherson 2004), beside a wide portfolio of different methods, procedures, hardware, including also robotic procedures, which were developed already and till now are under continuous optimization (Chayen 2003; Chayen and Saridakis 2008). Protein crystal growth is still considered to be an art, as the pathways protein crystal growth remain to some extend unpredictable (Gavira 2016; McPherson 2011).

Only recently latest biophysical methods and advanced diagnostic tools provided insights into early stages of the crystallization processes shed light towards understanding particular the nucleation process in more detail (Vekilov 2004; Schubert et al. 2017). In protein crystallization, a protein solution is typically brought into supersaturation by the presence of a precipitant, which is taking slowly water solvent molecules away from the protein surface and forcing in emergence of intramolecular protein–protein interactions, inducing in consequence the thermodynamically driven crystal nucleation followed by further crystal growth.

Beside purity and homogeneity of the protein solution the solution conditions at which supersaturation is achieved are the most important parameters, as they define a highly specific temporal pathway through the phase diagram (Gavira 2016).

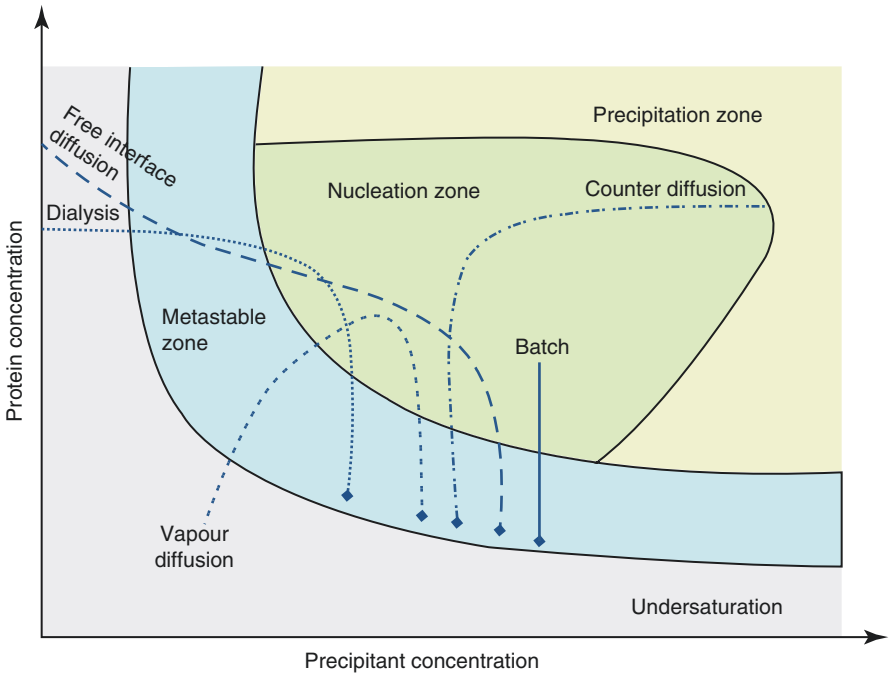

Fig. 2.1 Phase diagram demonstrating effects of protein concentration against precipitant concentration. The solubility curve divides two areas corresponding to the undersaturated and supersaturated state of a protein solution. The supersaturated area harbors the metastable, nucleation and precipitation zones

The phase diagram is commonly used to explain the crystallization process based on the Debye Hückel theory (Debye and Hückel 1923). As shown in Figs. 2.1 and 2.2 a supersaturation state can be achieved either rather straight, using the batch technique, or more smooth applying dialysis, vapor diffusion, or counter diffusion (McPherson 2004; McPherson and DeLucas 2015), shown in Fig. 2.2. The last two methods are the today most commonly used for standard screening experiments. The counter diffusion method, allowing to control and adjust precise and reproducible mass transport in protein crystal growth, was in recent years further developed and optimized for space experiments (Gonzalez-Ramirez et al. 2008).

On Earth density differences near growing crystals, produced by incorporation of protein molecules, solute and ions into the growing crystal, cause convective flows within the vicinity of growing crystals. These convective flows overlap with transport based on diffusion. On Earth the interaction and overlap of both transport phenomena, also shown in Fig. 2.3, determine the kinetics of crystal growth.

In this context, it is well known that the rate and kinetics of the overall mass transport is determining also the incorporation of impurities, such as protein aggregates, partially unfolded proteins etc., which can be present in crystallization solutions to some extend and influence the crystal quality and the final dimensions and form of a crystal. As convection flows are caused by heavy or more light fluids,

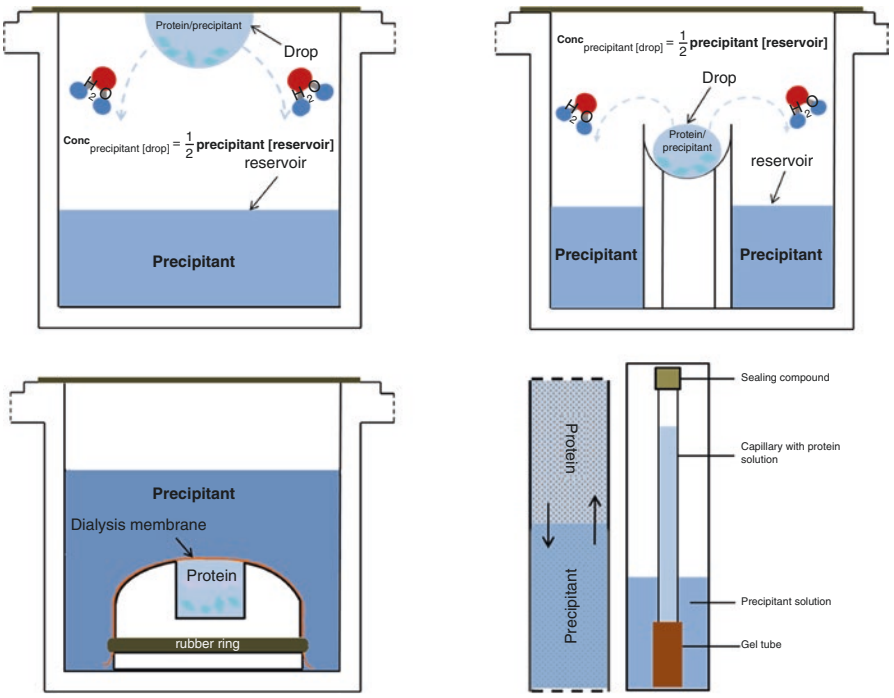

Fig. 2.2 Schemes of the most commonly used crystallization methods. *Top*, vapor diffusion, hanging and sitting drop. Below *left*, dialysis and *right*, counter diffusion

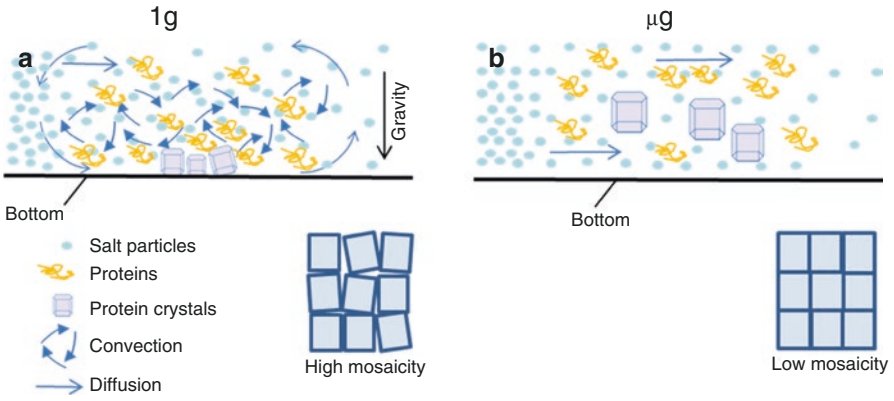

Fig. 2.3 Scheme (**a**) and (**b**) showing comparative mass transport under Earth and microgravity conditions and resulting crystal quality indicated by the mosaicity of the crystal lattice

which move or circulate in counter directions under normal gravity (1 g) they are somehow unfortunate and not really controllable, and they disturb to a certain extent crystal growth and crystal perfection. Therefore, crystal growth at 0 g or in a microgravity environment (10^{-3} to 10^{-7} g) instead, allows to minimize the convective flow

and associated mass transport resulting in an environment with mass transport based only on diffusion, as shown in Fig. 2.3b. As a result, crystals can grow with less incorporated impurities, substantial lower mosaicity and increased volume in comparison to their 1 g controls.

As mentioned before, already the first space experiment, performed by Littke and John in 1984 in the frame of the German TEXUS program (Littke and John 1986), confirmed that microgravity is an attractive environment to produce protein crystals with improved quality. In consequence, since early days of microgravity research growth of protein crystals and other biomolecules in microgravity is a distinct topic in international microgravity research activities. Different hardware and procedures were established to perform crystallization experiments on unmanned satellites, on space shuttle missions and space stations such as MIR and ISS. From early experiments till now considerable and continuous progress was made constructing and adapting crystallization hardware for microgravity experiments, considering the special environment of the spacecraft selected for the experiments. In principle, all crystallization methods mentioned before and shown in Fig. 2.2, applied for lab experiments for protein crystal growth, were adapted to individual space experiments. The following table is showing a summary of hardware most frequently used for protein crystallization experiments (Table 2.1).

The publication by Littke and John (1984) was well recognized by scientists working in the field of X-ray crystallography, followed by emerging new and further concepts to perform protein crystal growth in space. All concepts and following experiments were connected with the expectations that improved protein crystal growth in space will boost X-ray crystallography and structural biology. At the early time of space experiments, it was even considered that protein crystals can be obtained from sample suspensions, which even do not crystallize on Earth, due to the reduction before mentioned unfortunate convection, supporting smooth nucleation and further crystal growth. However already in the middle 1980s all crystallization experiments in space followed a same experimental plan, first the protein depended crystallization procedure was adapted to the flight hardware in the laboratories of the investigators. The final protein crystallization protocol was used to prepare and fill the flight hardware at the lab of the principle investigator, or at the launch site. In parallel the same number and a 1:1 replicate of the crystallization experiment was prepared for ground control experiments. Upon return all experiments and crystals obtained were first visually inspected and analyzed comparative to the ground control experiments. In a next step the samples were transported back to the home lab of the investigator for follow up comparative diffraction data collection and all steps of structure solution and refinement.

Protein crystallization experiments under microgravity conditions have resulted till now in more than 100 examples showing clear improvements in crystal quality via X-ray diffraction analysis, however also a number of experiments failed or did not show differences in space and ground control grown crystals. The majority of the crystal improvements resulted in improved resolution of the three-dimensional protein structures determined by X-ray crystallography.

Table 2.1 Protein crystallization hardware applied for microgravity crystallization experiments

Crystallization hardware	Crystallization method	Mission Space Shuttle	Year	References
Protein crystallization facility	Free interface diffusion/ liquid-liquid diffusion	TEXUS rocket	1984	Littke and John (1984)
VDA, Vapor Diffusion Apparatus	Vapor diffusion	STS-51D, STS-51F, STS-61B, STS-61C	1985–1986	Gonzalez-Ramirez et al. (2008), DeLucas et al. (1986)
CRYOSTAT	Liquid-liquid diffusion, free interface diffusion	STS-42	1992	Day and McPherson (1992)
PCF (protein crystallization facility)	Vapor diffusion	STS-37, STS-60	1991–1994	Gonzalez-Ramirez et al. (2008)
HH-DTC (Hand-Held Diffusion Test Cells)	Liquid-Liquid diffusion	STS-94	1997	Gonzalez-Ramirez et al. (2008)
APCF (Advanced protein crystallization facility)	Dialysis, vapor diffusion (hanging drop), liquid-liquid diffusion (free interface diffusion)	STS-95, ISS Mission 7A.1, STS 105, returned on Mission UF-1, STS 108	1993 onwards	Bosch et al. (1992) https://www.nasa.gov/centers/marshall/news/background/facts/apcf.html
PCAM (Protein crystallization apparatus for microgravity)	Vapor diffusion (sitting drop)	STS-62, STS-67	1994–1997	Gonzalez-Ramirez et al. (2008)
GN 2 (Gaseous Nitrogen-dewar)	Liquid-liquid diffusion	ISS Mission STS-110/8A, STS-111/UF-2, STS-71, STS-74, STS-76, STS-79, STS-81, STS-84, STS-89	1995–2002	Gonzalez-Ramirez et al. (2008)
DCAM (Diffusion-controlled crystallization apparatus for microgravity)	Dialysis	STS-76, STS-79, STS-81, STS-84, STS 89, STS-107, ISS, MIR	1996	Carter et al. (1999)

(continued)

Table 2.1 (continued)

Crystallization hardware	Crystallization method	Mission Space Shuttle	Year	References
CAPE (Canadian Protein Crystallization Experiment)	Liquid-liquid diffusion	Mir-24/NASA-6	1997	Schlagheck and Trach (2003)
GCF (Granada crystallization facility)	Counter diffusion	ISS, STS-105, STS-111, STS-113, STS-115	From 2001	Zegers et al. (2006)
HDPCG (High-Density Protein Crystal Growth)	Vapor diffusion	ISS Mission 8A, STS-100, STS-110, STS-111	From 2001	Rahman et al. (2015)
Modul-1	Free interface diffusion, liquid-liquid interface	ISS	From 2005	Smirnova et al. (2009)
SCDF (Solution crystallization diagnostic facility)	Batch, dialysis	ISS	From 2007	Pletser et al. (2006, 2008)
GCF-2 (Granada crystallization facility)	Counter diffusion	ESA-FOTON M-3 capsule	from 2007	Gonzalez-Ramirez et al. (2008)
PCDF (Protein crystallization diagnostics facility)	Batch, dialysis	ISS Mission 1E, STS-122	From 2008	Joannes et al. (2004), Pletser et al. (2009)
SCOF (Solution Crystallization Observation Facility)	Liquid diffusion	JAXA, ISS, STS-123/1J/A	From 2008	Yoshizaki et al. (2013)
DLR, SIMBOX	Counter diffusion	Chinese space mission Shenzhou-8	2011	Drebes et al. (2016)

As these examples demonstrate, the crystallization of organic macromolecules in microgravity is yet another example of how, in research, patience will usually pay off in the end. According to a review published in 2001 (Kundrot et al. 2001), experiments under microgravity have produced better crystals in about 20% of cases worldwide. If we leave out the substances that have been in space only once, the success rate rises to about 35%. If we include only those macromolecules that have been investigated in space more than four times, the success rate jumps to more than 60%. This again demonstrates that research needs patience and some staying power (Fig. 2.4).

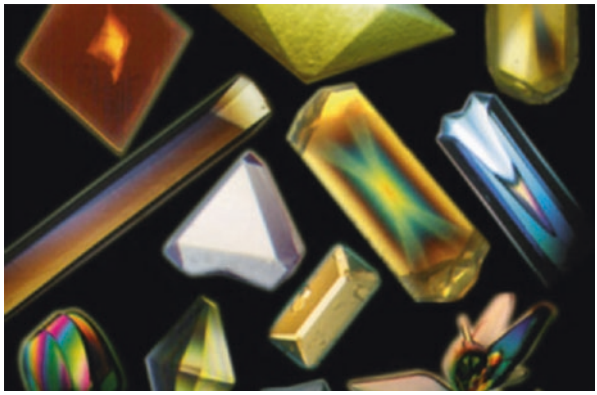

Fig. 2.4 Artist view of a collection of space-grown protein crystals (photo: NASA/Prof. McPherson, modified)

2.3 Early Successes of Structure Elucidation as Obtained in the German Space Life Sciences Program

2.3.1 The Structure and Function of Photo System I

Green plants and algae use photosynthesis to generate their own energy. With the help of sunlight, they convert carbon dioxide and water into sugar and oxygen. The basic chemical equation is very simple while the actual mechanism is extremely complex and has not yet been fully understood. Two major protein cofactor complexes, called photo systems I and II, are of major importance to the process as they ensure that the biological energy conversion process runs extremely effectively—with an energy yield amounting to almost 100%. For comparison: modern photovoltaic cells do not even reach 20%. The multisubunit PSI protein complex with its cofactors and pigments such as chlorophylls and carotenoids uses the energy of sunlight to convert carbon dioxide into oxygen and carbon in the form of carbohydrates, lipids, proteins and nucleic acids as the building blocks of life. PSII—likewise a multisubunit complex with various cofactors—uses the light energy to split water into hydrogen and oxygen for respiration.

It is these two photo systems whose structure, function and dynamics researchers worldwide are eager to work out and to understand in detail. Here again, microgravity is doing its bit. A first breakthrough for photo system I was achieved by scientists from Berlin's Technical University during the USML-2 shuttle mission in 1995. Crystals were grown on this mission whose volume was 20 times that ever reached for crystals grown on Earth. Based on these crystals an improved structural model of photo system I was developed at 4 Ångström (or 10^{-4} µm). This model rendered important functional parts of this large complex visible for the first time (Krauß et al. 1996).

Further experiments conducted during the 1998 STS-95 shuttle mission showed the nucleation rate to be significantly lower in weightlessness, leading to the formation of

larger, almost perfect crystals. Overall, crystals grown under microgravity conditions featured a significantly better resolution and fewer defects than comparable crystals grown on the ground (Klukas et al. 1999a, b).

2.3.2 The Crystallization of Archaea Surface Proteins

So-called S-layers are probably the evolution's first cell wall structures that came into being some three billion years ago. They consist of certain crystalline proteins on the surface of archaebacteria. The name of these organisms is due to the fact that they occur in hostile habitats similar to that of the early Earth. Thanks to their S-layer they have an enormous resistance to heat, extreme pH values and high salt concentrations.

During shuttle flight STS-95 in October 1998, a team of scientists from the universities of Ulm and Mainz together with their Belgian colleagues succeeded for the first time in growing crystals of the S-layer glycoprotein of one of these archaebacteria, *Methanothermus fervidus* (Evrard et al. 1999). Following their return to Earth, the crystals were examined by X-ray crystallography. At a resolution of 3 Ångström it became possible to clearly identify some of the crystals' parameters including their lattice constants and space group (Claus et al. 2002). In a series of experiments conducted on the ISS between June and October 2002, researchers were able to grow S-layer crystals of another organism, *Bacillus sphaericus*, at a resolution of 1.9 Ångström.

These experiments have been a great step forward towards understanding the structure of the S-layer. It is hoped that they will open up new insights into the survival strategies of these organisms and the underlying molecular mechanisms. This knowledge is not only important for science but might also open up options for new applications. Actually, the improved knowledge of S-layer structures has already found its way into the development of ultra-filtration membranes and other molecular nanotech applications.

2.3.3 Bacteriorhodopsin: A Promising Compound for Biotechnological Applications

Bacteriorhodopsin is the major photosynthetic protein of archaea such as *Halobacterium salinarium*. It converts the energy of green light—wavelengths between 500 and 650 nm—into an electrochemical protein gradient, which in turn is used for ATP production by the enzyme ATP synthase. It functions as a light-driven proton pump, transporting protons out of the cell. Bacteriorhodopsin has come into focus of much interest roughly 30 years ago, since its reversible light-triggered color change has allowed to develop biotechnological applications e.g. in

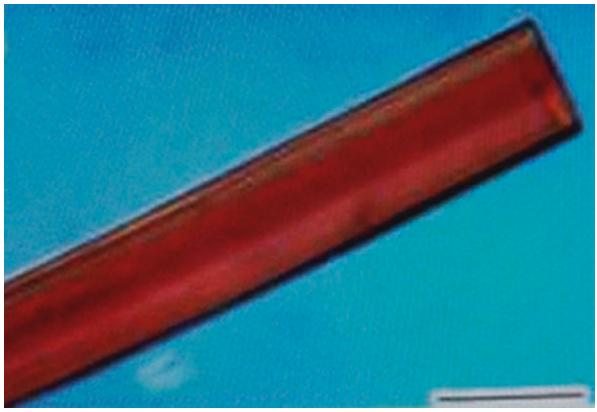

Fig. 2.5 Bacteriorhodopsin crystal grown during shuttle mission STS-95 (Zörb et al. 2002)

optical information recording (for an early review see Oesterhelt et al. 1991; Hampp 2000). Therefore, it is not surprising that elucidation of the structure and function of bacteriorhodopsin has got much attention.

Also, attempts were made to increase the size and perfectness of bacteriorhodopsin crystals by experiments under microgravity conditions. Indeed, experiments during the space shuttle flight STS-95 and on the Russian space station MIR in the late nineties demonstrated that large needles of the molecule could be grown (Fig. 2.5) that were not only larger in size, but also more homogenous and thus superior to those of the parallel ground controls (Zörb et al. 2002). Unfortunately, no further experiments were performed due to the retirement of the scientists, so that the final goal to achieve crystals with dimensions of 1 cm by 1 cm by 1 cm—desirable for successful application on ground—could not be achieved.

2.3.4 Mistletoe Lectin as an Agent in Immune Stimulation and Cancer Treatment

Bare trees bearing strange globular objects are a common sight along the railway lines as you travel from Cologne to Paris on a winter's day. The globular shapes are mistletoe (*Viscum album*), an evergreen plant living in symbiosis on trees (Fig. 2.6). For many centuries people have used it as a medicinal plant. We now know that the main component in mistletoe extracts, frequently used to strengthen the human immune system and in cancer treatment, is a compound called mistletoe lectin-I. Its precise mode of action is still to a large extent unexplained. A closer investigation of its three-dimensional structure is hoped to provide clarification. For some years now, this question has been Professor Betzel's area of research at Hamburg University (see Chaps. 3 and 4). Experiments carried out on the ISS in 2001, 2002 and 2006 provided crystals that were suitable for an improved structural analysis (Krauspenhaar et al. 2002; Meyer et al. 2008). Thus, it became possible for the first

Fig. 2.6 Mistletoe in poplar trees

time to explain what goes on in their active centers. The ribosome blocking protein mistletoe lectin-I (ML-I) is built from two separate protein chains, called A and B. Scientists today assume that subunit B is able to recognize certain sugar molecules on the membrane of the cell to be attacked, and thus helps subunit A to penetrate into that cell. This process then inhibits the cell's ribosome activity and ultimately leads to the death of what could be a cancer cell.

ISS-borne experiments also gave a clear indication that a wide variety of galactose and lactose sugar chains can adhere to the high-molecular protein complex (Mikeska et al. 2005). This also explains why crystals grown under regular laboratory conditions are of only moderate quality, limiting the possibility of an exact structural/functional analysis. Thanks to the space-borne experiments and accompanying investigations the necessary groundwork has now been done to improve the pharmaceutical application of this protein.

These results are a good example of how basic medical research can benefit from a detailed investigation of the structure of organic macromolecules and from understanding important molecular phenomena. The knowledge gained can be put to subsequent use in the development of specific inhibitors or in optimizing molecules which can then be fabricated in large quantities using methods of modern molecular biology.

2.3.5 Mirror-Image RNA Molecules

Ribonucleic acid, or RNA for short, provides the link between the DNA's genetic information and the proteins assembled by ribosomes as a result. Professor Erdmann and his team at Berlin Free University (FU Berlin) have focused research activities for many years on investigating the structure and crystallization of RNAs as well as their protein complexes. The starting point of their investigation was the ribosomal 5S rRNA, an important component of the ribosomes at which protein synthesis takes place. Crystals produced on shuttle flight STS-95 and on a 4 months' worth of experiments on board the ISS in 2001 have resulted in a detailed structure of domain B of the 5S rRNA (Vallaza et al. 2002; see Fig. 2.7). For the first time a diffraction pattern of the 5S rRNA/L18 protein complex was obtained.

Further experiments conducted in cooperation with the pharmaceutical company NOXXON AG on the ISS in 2002 led to the first successful crystallization of mirror-image RNA (Vallaza et al. 2004). The advantage of mirror-image nucleic acid compared to 'natural' RNA molecules lies in their long life in the human blood. This makes them particularly suitable for an effective treatment of tumors or viral infections such as AIDS. In addition, thanks to their great stability they can be chemically synthesized in large quantities and high levels of purity.

However, in order to understand the function of these molecules it is mandatory to understand their structure. One main focus of the ISS experiments was analyzing the interaction between nucleic acids and water molecules. The exact array of water molecules within the helix and its surroundings is important for the maintenance of the spatial structure of RNA. The model of 5S rRNA obtained as a result of these experiments opens up new insights into the interaction between antibiotics and ribosomal RNAs, thus permitting the development of more effective drugs.

Further experiments were conducted on the ISS in late 2008 and in October 2009. Researchers are hoping for further structural details of these important molecules. The ultimate expectation of the structural analysis of RNA samples was to provide a better understanding of ribosomal functions, hoping that the results will find their way into molecular medicine. This is where we reach a full circle: more than 20 years ago Professor Erdmann together with Professor Ada Yonath and Professor Heinz-Günter Wittmann laid the groundwork for the investigation of ribosomal structures.

2.4 Perspectives for Protein Crystallization in Space

What does the future hold in store? It is obvious that, as things have been going so well, the crystallization of organic macromolecules under microgravity conditions will continue to be part of Germany's space program, and those of space agencies worldwide, too. It would be wrong, though, to count on easy answers and to place excessive expectations in the benefit of weightlessness, as people did in the

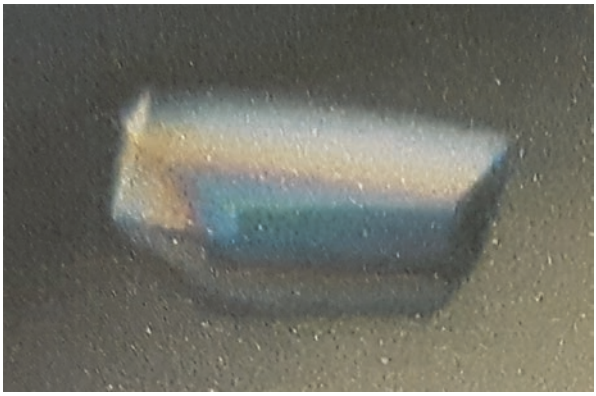

Fig. 2.7 5S RNA crystal grown during IML-2 shuttle mission STS-65 applying the APCF hardware (Förster et al. 2011)

program's early days. After all, gravity is only one of about 20 factors influencing the crystallization process. Yet, as part of a combined effort with Earth-based research, experiments in space will continue to deliver the desired progress. Also, as NOXXON GmbH and RiNA GmbH—both founded in Berlin in the context of the before mentioned biotechnology and structural biology investigations—have demonstrated, we can expect further start-up companies to be successfully placed in the market in the area of drug discovery and design.

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Chapter 3
Protein Crystallization on the International Space Station ISS

Christian Betzel, Arayik Martirosyan, and Günter Ruyters

Abstract Already early protein crystallization experiments in space indicated that more extended crystallization periods, beyond the flight durations of shuttle missions or unmanned orbiters, will be beneficial for the majority of microgravity protein crystal growth experiments. Beside preceding intensive efforts to adjust and optimize crystallization conditions to meet the microgravity time span of orbiters flight duration, video imaging of some experiments showed that the crystallization process was not finalized at the end of the mission. And a number of experiments performed on MIR, prior to availability of ISS, confirmed potential advantages applying extended microgravity crystallization periods, also knowing that due to some crew activities the microgravity on ISS may not sustain a 100% convection free environment in the crystallization hardware. Considering this fact as a minor restraint and knowing that most biomolecules require and appreciate growth periods longer than the duration of a typical shuttle mission of 7–10 days, opportunities to perform crystallization experiments on ISS are very attractive.

Keywords Crystal Quality • Crystallization Methods and Techniques • Microgravity Experiments

3.1 Hardware Constructed and Adapted to ISS Crystallization Experiments

Within the early construction and commissioning phase of ISS already several crystallization experiments were performed and data were reported showing that crystal growth in microgravity on ISS results in high, up to significant improvements, reflected in terms and parameters like the Rmerge, B-value, mosaicity, higher quality electron density and identification of more solvent atoms (Barnes et al. 2002; Berisio et al. 2002; Vallazza et al. 2002).

Stimulated by the positive results first hardware concepts were discussed to construct and install a compact X-ray data collection unit on ISS, including all required sample and crystal handling apparatuses. The concept to utilize protein crystals

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grown on ISS straight was in principle profound, because collecting data on ISS would also avoid unfortunate mechanical stress for microgravity grown crystals they may face during transport back to Earth and during further transport back to the lab of the principle investigators. However, at the same time it needs to be considered that high intensive synchrotron radiation is always beneficial for diffraction data collection on Earth. Along these objectives and considerations scientists and engineers at the center for structural biology and engineering (CBSE) at the University of Alabama (UAB) designed a compact and complete crystallographic laboratory for the International Space Station, including a crystal growth facility. The system was supposed to host a variety of crystallization hardware systems available at this time, a crystal harvesting and cryopreservation robotic system, a diffraction unit with X-ray generator in combination with a particular high focusing X-ray optics and CCD detector to characterize crystals and being capable to collect complete X-ray diffraction data sets. All components of this unit were supposed to be operational utilizing only minimal crew time. The entire system was constructed at UAB/CSBE, however not put in operation till now (DeLucas et al. 1999, 2000).

Nevertheless, as worldwide research activities in structural biology and structure based drug design, in academia and industry, were extending rapidly in the last 15 years also the demand to obtain more insights about crystallization phenomena was increasing as well, because high quality crystals are mandatory. In this context, the tremendous opportunities to take advantage of extended crystallization periods on ISS merged with efforts to implement and adapt diagnostic tools to already existing crystallization systems. For example, the APCF hardware was modified and upgraded for ISS and a system designated as PCDF (Protein Crystal Growth Diagnostic Facility) was particular designed and constructed to harbor a video recording system (Carotenuto et al. 2001), diagnostic tools like a Mach-Zehnder-Interferometer and Dynamic Laser Light Scattering (Pletser et al. 1999, 2006, 2009). Mach-Zehnder-Interferometry is most ideal to analyse fluid transport and its alteration around growing crystals and to quantitate the concentration of macromolecules in depletion zones around growing crystals (Tanaka et al. 2006). In 2013 details were reported about the SCOF, the Solution Crystallization Observation Facility, employing also Mach-Zehnder Interferometry to measure crystal growth rates during protein crystallization experiments on ISS (Yoshizaki et al. 2004, 2013). Dynamic Light Scattering (DLS) was integrated in several diagnostic systems to obtain information about the homogeneity and quality of protein suspensions and to obtain insights about the early events of protein crystal growth (Stapelmann et al. 2001). Most crystallization experiments performed by utilizing these analytical tools were carried out under microgravity conditions and for control also on ground to obtain maximum insights about the crystallization process.

McPherson and co-workers constructed and reported in 1999 details about the protein crystal growth apparatus (OPCGA), capable to analyze the fluid environment around growing crystals and capable to identify and monitor quasi-stable depletion zones around growing crystals in space (McPherson et al. 1999). It is of interest to mention, that OPCGA was designed and constructed to follow and score particular the incorporation of impurities in growing crystals. Impurities cause lat-

tice defects, dislocations and local disorders in crystals and overall disturbing the diffraction quality by reducing the amount of unit cells contributing to the useful Bragg-intensities. At the same time the background and diffuse scattering is increased, disturbing data reduction and processing. Analyzing and understanding the incorporation of impurities came back in focus of research activities on ISS in 2013 (see Chap. 8), because with commissioning of Free Electron Laser Sources the demand to produce most perfect protein crystals is growing again.

To analyze counter diffusion protein crystallization experiments a special microscope, the protein microscope for the International Space Station, PromISS, was installed. Digital holography as method was applied to visualize the crystallization process, to score crystal growth and also to monitor crystal movement during growth in capillary counter diffusion and batch crystallization experiments (Zegers et al. 2006). PromISS allowed for the first time to identify and analyze the moment crystals appear, the crystal growth rate and crystal movements. Zegers et al. reported details about the instrument and its application; however, they also mention some unfortunate problems like temperature instabilities and vibrations on ISS, causing a shift of the interferometric rings. Nevertheless, crystals of a trio phosphate isomerase were obtained which diffracted to 1.7 Å resolution, compared to Earth grown counterparts diffracting only up to 2.5 Å.

Until now the particular and unique experimental opportunities on ISS stimulate the design and construction of new diagnostics tools to obtain further insights about the crystal growth nucleation process, concentration gradients near growing crystals and their shapes and magnitudes. In summary, the International Space Station opened from the beginning up to now opportunities for innovative protein crystal growth experiments. Representative experiments successfully performed on ISS and published are summarized in Table 3.1. A few examples and results of such experiments are highlighted and summarized in the following section.

Table 3.1 Selected and representative proteins crystallized on ISS

Protein	Crystallization method/techniques	Year/time period	Mission	References
Human triosephosphate isomerase	Counter diffusion	2001–2005	ISS	Kinoshita et al. (2005)
Thermus flavus 5S rRNA helix B	Vapor diffusion	April 2001 to August 2001	ISS 6A, STS-100, STS-105	Vallazza et al. (2002)
Mistletoe lectin I from *Viscum album* in complex with adenine monophosphate	Vapor diffusion	2002, November, 110 days	ISS 6A, STS-113	Krauspenhaar et al. (2002); Edward and John (2005)
Apocrustacyanin C(1)	Vapor diffusion	2002	US Space shuttle, ISS mission	Habash et al. (2003)
Myoglobin triple mutant Mb-YQR [L(B10)Y, H(E7)Q and T(E10)R]	Vapor diffusion	2001, 2002	ISS 6A, ISS 8 A	Miele et al. (2003)

(continued)

Table 3.1 (continued)

Protein	Crystallization method/techniques	Year/time period	Mission	References
Manganese superoxide dismutase	Vapor diffusion	December 2001 to April 2002	ISS	Vahedi-Faridi et al. (2003)
Hydrogenase maturation factor HypF N-terminal domain	Vapor diffusion	April 2002	ISS 8A/ STS110	Ponassi et al. (2011)
Liver Basic Fatty Acid-Binding Protein, Chicken liver basic Fatty Acid-binding protein complexed with cholic acid	Vapor diffusion	2001	STS-100, ISS-6A	Nichesola et al. (2004)
Hematopoietic prostaglandin D synthase	Counter diffusion	January–April 2004, 13 weeks	NASDA-GCF#3, ISS	Tanaka et al. (2011)
Hen egg lysozyme, Carboxypeptidase B	Free-interface-diffusion	April–October 2005	ISS 11	Smirnova et al. (2009)
Recombinant human insulin	Free-interface-diffusion	October 2005 to April 2006	ISS12-13	Smirnova et al. (2009)
Thermotoga maritima triose phosphate isomerase	Counter diffusion	2007	ISS	Evrard et al. (2007)
Archaeal transcription termination factor NusA	Counter diffusion		ISS, JAXA–GCF project	Tanaka et al. (2007)
Mistletoe Lectin I (ML-I)	Counter diffusion	2005	ISS	Małecki et al. (2012)
Mistletoe Lectin I in complex with Zeatin	Counter diffusion	2005	ISS	Meyer et al. (2008)
Uridine phosphorylase from *Shewanella oneidensis* MR-1	Free-interface-diffusion, counter diffusion	2012	ISS, JAXA	Safonova et al. (2012)
Mouse Lipocalin-Type Prostaglandin D Synthase	Counter diffusion	August to October 2007, 11 weeks	ISS, Russian Module-1, JAXA	Inaka et al. (2011)
Recombinant Formate Dehydrogenase from *Arabidopsis thaliana*	Vapor diffusion	2006–2009	ISS, Russian Module	Shabalin et al. (2010)
Phosphopantetheine Adenylyltransferase from *Mycobacterium tuberculosis* in complex with Coenzyme A	Vapor diffusion Counter diffusion	2006–2009	ISS, Russian Module	Timofeev et al. (2010, 2012b)

Table 3.1 (continued)

Protein	Crystallization method/techniques	Year/time period	Mission	References
Human epidermal growth factor receptor	Counter diffusion	2011	ISS	Yoshikawa et al. (2013)
Phosphopantetheine adenylyltransferase (PPAT) from *Mycobacterium tuberculosis* in the apo form, Phosphopantetheine Adenylyltransferase in comlex with dephosphocoenzyme A	Counter diffusion	2012	ISS, Russian Module	Timofeev et al. (2012b)
Phosphopantetheine adenylyltransferase (PPAT) from *Mycobacterium tuberculosis* in the apo form, Phosphopantetheine adenylyltransferase (PPAT) in the complex with ATP	Counter diffusion	2012	ISS, Russian Module	Timofeev et al. (2012a)
Thymidine Phosphorylase from E. coli in Complex with 3′-Azido-2′-Fluoro-2′,3′-Dideoxyuridine	Counter diffusion	2012	ISS, Russian Module, JAXA	Timofeev et al. (2013a)
Carboxypeptidase T from *Thermoactinomyces vulgaris* in Complex with N-BOC-L-eucine	Counter diffusion	2012	ISS, Russian Module	Timofeev et al. (2013b)
Thermostable T1 Lipase	Counter diffusion	2014	ISS, JAXA	Mohamad Aris et al. (2014)
Zn-insulin	Counter diffusion, free-interface diffusion	2014	ISS, Russian Module	Strelov et al. (2014)
Porcine carboxypeptidase B	Counter diffusion	2015	ISS, Russian Module, JAXA	Akparov et al. (2015)
Phosphoribosyl pyrophosphate synthetase from *E. coli*	Counter diffusion	2016	ISS, Russian Module	Timofeev et al. (2016)
D-tagatose 3-epimerase C66S from *Pseudomonas cichorii* in complex with 1-deoxy L-tagatose	Counter diffusion	2013–2016	ISS, JAXA-PCG	Yoshida et al. (2016)

3.2 Long Term Crystallization Experiments: Results, Advantages and Considerations

Barnes et al. (2002) published a crystal growth experiment of thaumatin carried out in 2000 for 2 months resulting in superior diffraction quality of crystals grown in microgravity environment on ISS and providing diffraction data to a resolution limit at this time of 1.28 Å, compared to ground control crystals diffracting to 1.47 Å. Crystals of a superoxide dismutase grown between December 2001 and April 2002 on ISS were reported to have even a approximately 80 times higher volume than Earth grown crystals, permitting data collection to 1.26 Å resolution (Vahedi-Faridi et al. 2003; Vergara et al. 2002). Further, experimental periods of 3 month and 20 days on ISS were used to crystallize collagen-like polypeptide $(PPG)_{10}$ applying the APCDF hardware provided by ESA (Bosch et al. 1992), allowing at this time to perform a bunch of comparative experiments and allowing to screen a wide range of conditions (Berisio et al. 2002). However, for highly extended crystallization periods performed on ISS it was also reported that some crystals suffered from ageing, an effect also sometimes observed for Earth grown crystals. If the process of crystal growth is completed and protein solution as well as precipitant reached equilibrium some grown crystals tend to decay after a period of time (McPherson 2004).

Joining international research collaborations, scientists from Germany could take advantage of extended crystallization experiments on ISS. For example, a ribosome inhibiting protein was crystallized during the ISS assembly mission 6A in 2001. Crystals were grown in complex with selected ligands during a period of 110 days. For these experiments, the high density protein growth system (HDPCG) provided by the University of Alabama (UAB) was applied. X-ray suitable crystals obtained provided diffraction data up to 1.9 Å resolution (Krauspenhaar et al. 2002). The same mission was hosting experiments to crystallize Helix B of the ribosomal 5S RNA (Fig. 3.1). Crystallization procedures and structural data and results were described by Vallazza et al. (2002), highlighting for the first time structural data

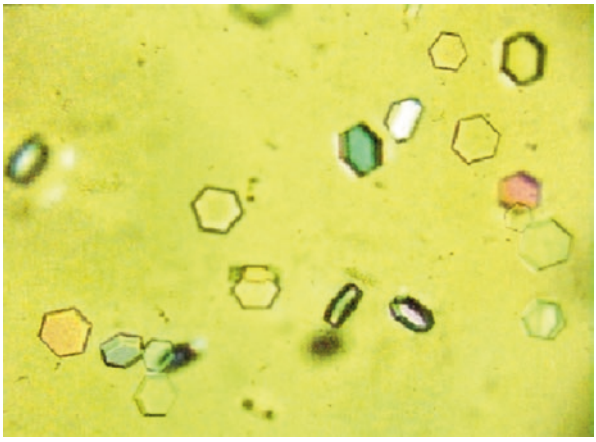

Fig. 3.1 Space grown crystals of the 7 bp 5S RNA Helix B with dimensions of approximately $0.1 \times 0.1 \times 0.02$ mm (Vallazza et al. 2002)

revealing the coordination of non Watson Crick base pairing and internal solvent water compensating and retaining the H-bond network within base pairs of a natural RNA helix.

Structural data provided insights about the helical stability and distinct structural motifs and binding sites for proteins. For these experiments, during mission ISS 6a, also the HDPCG and the Commercial Incubator-Refrigerator module (CRIM-M) were used.

As mentioned before, the delay time after preparation of crystallization suspensions and placing them in the compartments of the crystallization hardware till the activation of the experiment is most critical, and should be as short as possible. Unfortunately, in the past some ISS microgravity experiments suffered from effects caused by extended transport and delay periods, as for example in protein solutions at high concentrations, required for a crystallization experiment, proteins aggregated or precipitated. And sometimes such situations were combined with unfortunate effects of rough transport or/and by a launch delay of the transport orbiter. Such data and experiences are mostly not published, however shared and exchanged within the protein crystallography community. To overcome such problems, biased also by continuous increasing security requirements within international transport and courier companies, required to send biological samples to the locations and labs the crystallization hardware is prepared for the ISS experiments, the crystallization hardware systems were further developed and optimized till now.

One crystallization system which can compensate and tolerate to a rather high extend delay and transport time to ISS is the counter diffusion method in capillaries, shown in Chap. 2, Fig. 2.2. This method is well established since the early time of protein crystal growth and was re-invented and optimized by Garcia-Ruiz and coworkers (Gonzalez-Ramirez et al. 2008), introducing also the option to use gels, which in part can reduce convection in thin capillaries. The hardware was named after as Granada Crystallization Facility (GCF) or Granada Crystallization Box (GCB). The counter diffusion method was also implemented in the APCF hardware and used for several protein crystallization experiments on ISS, for example during the ISS taxi flight missions Odissea and Cervantes in 2002 and 2003. Evrard and coworkers analyzed in detail ground grown crystals versus space grown TIM crystals, obtained during this two missions, and data again confirm that space grown crystal showed overall higher quality via parameters I/σI, Rmerge and mosaicity, beside an increase in diffraction power (Evrard et al. 2007). Actually the authors report that they did not expect such a significant improvement, because also they observed in a further and contemporaneous experiment on ISS g-jitter and residual acceleration causing also substantial crystal movements and convective flows (Zegers et al. 2006; Simic-Stefani et al. 2006).

Because of its convenient handiness over the last years, the counter diffusion method became the most popular used method to crystallize proteins on ISS. Consequently, the Japan Aerospace Exploration Agency (JAXA) introduced with some modification compared to the originally capillary counter diffusion methods the JAXA crystallization Box (JCB) for ISS space experiments (Sato et al. 2006). JCB contains six rather thick wall glass capillaries with 0.5 mm diameter and 60 mm

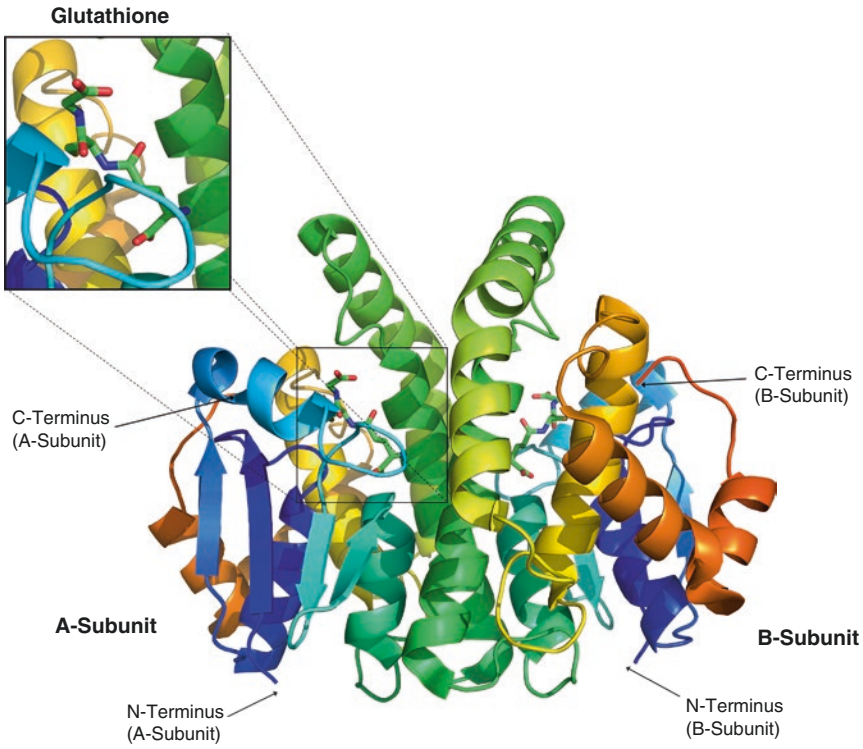

Fig. 3.2 Cartoon representation of one enzyme dimer (subunit A and subunit B) of the human hematopoietic prostaglandin D synthase (pdb code: 1IYI_2) with bound glutathione (stick presentation) in the binding site (Tanaka et al. 2011)

length, which are placed in hermetically closed plastic boxes. In 2013, JAXA published statistics summarizing experiments performed between 2002 and 2012 (Takahashi et al. 2013).

Overall 14 experiments were performed on ISS within this period. In 2008, the JCB setup was established in the Protein Crystallization Facility (PCRF) of the Japanese Experiment Modul named Kibo; approximately 500 proteins were crystallized mainly by Japanese, Russian and Malaysian scientists (Takahashi et al. 2013). And in 2015, JAXA established industrial crystal growth experiments in the Commercial Protein Crystal Growth unit (CPCG) on ISS.

Examples worth to mention are the following crystallization experiments, which utilized the JCB hardware: Urade and coworkers reported improved crystals of a Prostaglandin D Synthase obtained during a ISS mission in 2007, which were used for data collection to analyze the structure (Fig. 3.2) (Tanaka et al. 2011; Inaka et al. 2011).

Superior crystals of a ribosome inhibiting protein in complex with a phytohormone, shown in Fig. 3.3, were grown during 3 month on the JAXA-GCF space mission No. 6 in 2006. Structural data and a comparative analysis of diffraction data were published in 2008 (Meyer et al. 2008).

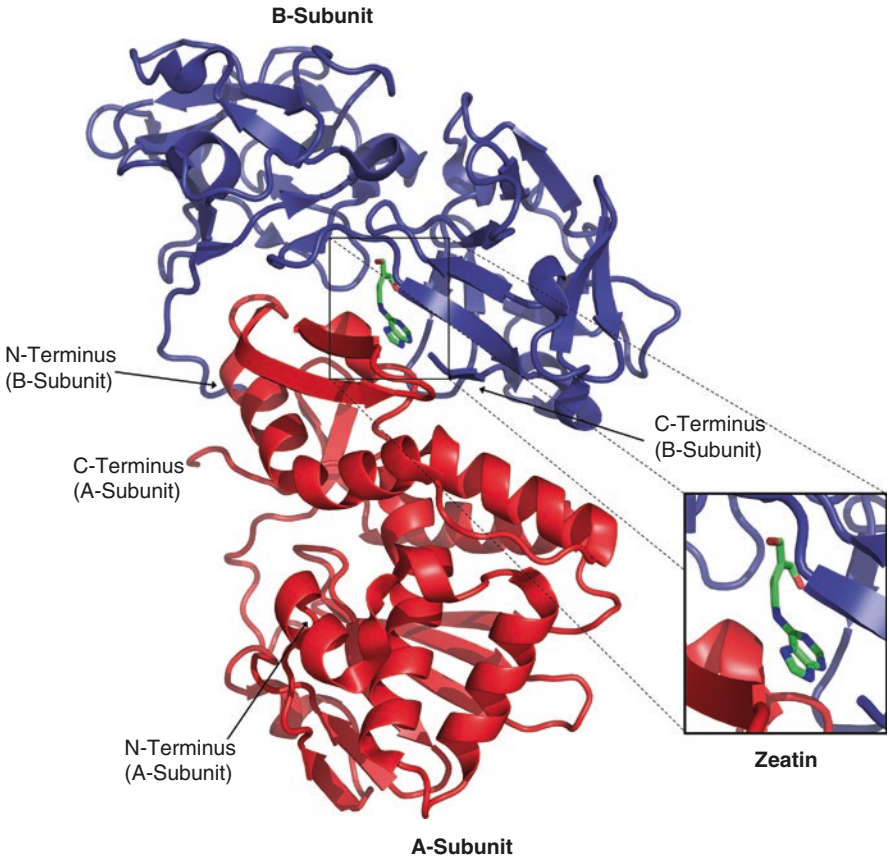

Fig. 3.3 Cartoon plot of mistletoe lectin I, ribosome inhibiting protein, in complex with zeatin (pdb code: 3D7W). Subunit B is shown on top and subunit A below. The zoomed zeatin binding cavity is located almost at the center of the protein (Meyer et al. 2008)

Kuranova and coworkers performed several microgravity crystallization experiments particular within the Russian Segment of the ISS (Smirnova et al. 2009; Kuranova et al. 2011) and reported in 2012 the successful crystallization of the adenylylferase from *Mycobacterium tuberculosis* applying the JAXA JCB during a mission in 2011 (Fig. 3.4). Diffraction data obtained showed up to 0.4 Å higher resolution compared to diffraction data obtained for Earth grown crystals (Timofeev et al. 2012a, b).

In summary, all published data highlight the tremendous advantage of long duration crystallization experiments and opportunities to perform diagnostic experiments (Strelov et al. 2014). However, they emphasize as well that crystal growth conditions need to be adapted and optimized to hardware and duration of the experiment as possible to exploit the potential of ISS for such experiments. After a period of collecting experience in performing crystallization experiments on ISS more interesting data and publications will certainly emerge in future.

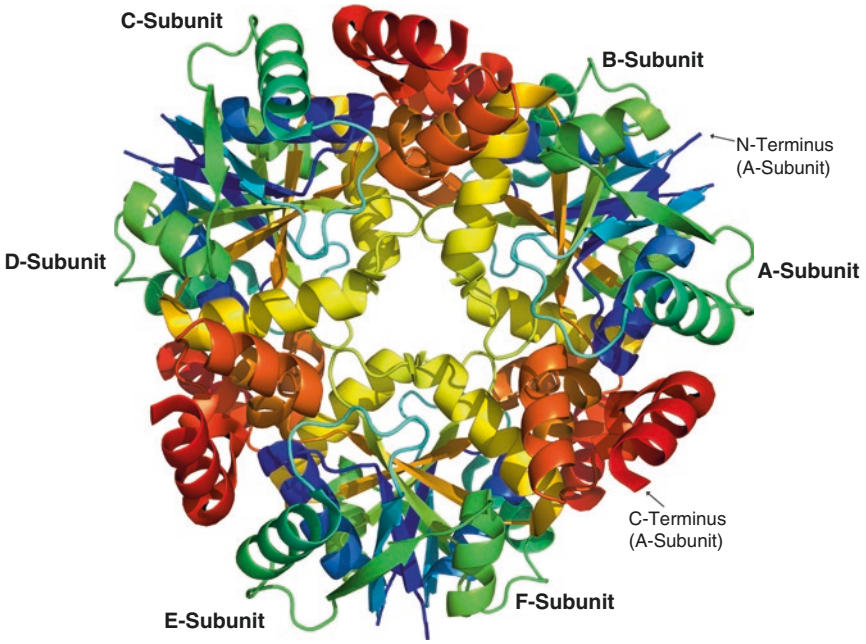

Fig. 3.4 Cartoon representation of the apo form of phosphopantetheine adenylyltransferase from *Mycobacterium tuberculosis* shows a homohexamer (chain A to chain F), which is composed of two trimers

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Chapter 4
Drug Design

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Abstract X-ray crystallographic data are today mandatory for drug discovery and are essential within the iterative process of drug design. Microgravity grown crystals of potential drug target proteins or complexes of drug target proteins with selected compounds supported the design and development of new generations of pharmaceuticals, which were forwarded to clinical trials to treat chronic and infectious diseases such as T-cell lymphoma, HIV, psoriasis, stroke and other cardiovascular complications, influenza and rheumatic arthritis. Results derived from crystals grown under microgravity conditions also contributed, for example, to the understanding of drug cancer- cell interactions.

Keywords Three-dimensional Structures • X-ray Analysis • Drug Design Investigations

4.1 Protein Crystallography and Drug Discovery

As emphasized in the chapters before, crystallography is till now the most suitable and most powerful technique to determine the three-dimensional structure of biological molecules at high resolution. Crystallographic studies of proteins and nucleic acids and their complexes revealed till now structural data and coordinates of more than 128,000 biomolecules, deposited in the Protein Data Bank (www.rcsb.org, Berman et al. 2000). Three-dimensional structures provide pivotal information to understand how macromolecules operate in biological systems; therefore, structural data obtained from crystallographic investigations are mandatory for industry and academic research institutions working in the field of protein engineering to optimize and adapt biomolecules towards particular applications in biotechnology; and particular high resolution structural data are crucial for pharmaceutical industry and research institutions performing drug design investigations. Particular pharmaceutical companies utilize protein crystallization and X-ray analysis during the

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early phases of the drug discovery process, prior to the pre-clinical phase, to identify ways to block or enhance activities of vital protein targets in diseases (Bedell 1992; Ciociola et al. 2014). Protein crystallography is the most suitable method to identify and further validate for example a receptor region, the molecular area where potential drugs should bind, considering also surrounding solvent molecules (Blundell et al. 2002).

Within initial working steps also bioinformatics approaches need to be considered and to be included to search for and to identify compounds, which can bind to a target receptor site (Klebe 2006). In most cases, it is either an active site and substrate binding region of an enzyme, or the ligand binding site of a membrane associated receptor. Within an iterative process, complex structures of the target protein and drug compound will be prepared in solution, crystallized for X-ray diffraction data collection and analyzed in three-dimensions at highest possible resolution to obtain first lead information how the ligand is binding and how to modify a potential drug compound further to gain most suitable binding constants. Within this iterative procedure, the number of interactions between receptor and ligand will ideally be maximized, however also considering stability of the compound and its water solubility (Erlanson et al. 2000).

Interactions which are in the focus of such investigations and optimization procedures are H-bonds and Van der Waals contacts with distances between 2.7 and 3.3 Å and 3.3–3.7 Å, respectively. Further, ionic interactions and interactions enabled by surrounding ions need to be considered (Creighton 1992). Also, information about bound solvent atoms and ideally about the entire hydration shell is required as well, as tight bound water clusters are competing with compounds supposed to bind to an active site region, compounds which have at the beginning of the iterative drug design process often a relative low water solubility (Kuntz et al. 1999).

As mentioned before, to perform drug discovery investigations structural information at highest possible resolutions is required to identify and analyze all interactions in detail and considering also the crystallographic occupancy of bound compounds, which in many cases is also less than 1.0 (Drenth 1994; Blundell and Johnson 1976). Therefore, it is today commonly accepted that even improvements in resolution of 0.3–0.5 Å have a significant impact on the ability to design and optimize drug compounds and to obtain more insights about the structure, function and dynamics of the targeted biomolecules. Such improvements in resolution can mainly and in most cases only be achieved by improving the crystal quality or crystal size. Consequently crystallization under microgravity conditions is highly attractive for structural investigations within the process of drug design (Pool 1989; DeLucas et al. 1986, 1989, 1999; DeLucas 2001).

4.2 Impact of Microgravity Crystallization on Structure Determination and Drug Design

Beside considering the continuous methodical and technical progress in all working steps of protein crystallography in the last 20 years, mainly achieved by the development of more sensitive X-ray area detectors and the application of third

generation synchrotron radiation sources and recently also X-ray Free-Electron Lasers for diffraction data collection, the crystal quality remains to be the most important parameter within X-ray structure analysis. Crystal size and internal order determines the resolution of a structure analysis and thus the molecular details which can be used for interpretation, as highlighted before. As the likelihood to obtain higher quality crystals for X-ray analysis in a microgravity environment was reported since the early experiments of Littke et al. till now (Littke and John 1984, 1986; Ng 2002; DeLucas et al. 2002; Terzyan et al. 2003; Chayen and Helliwell 2002; Akparov et al. 2015; Drebes et al. 2016) a substantial number of these experiments was performed in context of drug discovery and design investigations. Selected examples of microgravity crystallization experiments performed in terms of drug design investigations are summarized in Table 4.1.

The corresponding microgravity crystallization experiments carried out in terms of drug discovery investigations can be divided in crystallization experiments of

Table 4.1 Microgravity crystallization experiments in context to drug design investigations

Protein	Space Mission	Function/Target	Reference
C-reactive protein	STS-61C	Binds to the phosphocholine expressed on the surface of dead or dying cells and some bacteria	DeLucas et al. (1986)
Human serum albumin	STS-61C, STS-42	Maintains the oncotic pressure and transports for example fatty acids and thyroid hormones as well as unconjugated bilirubin	DeLucas et al. (1986, 1994)
Purine nucleoside phosphorylase	STS-61C	Metabolizes inosine towards hypoxanthine and guanosine in guanine	DeLucas et al. (1986)
TET repressor	Chinese re-entry capsule	Involved in bacterial resistance again antibiotics	Erdmann et al. (1989)
Ribonuclease S	Chinese re-entry capsule	Catalyzes the degradation of RNA into smaller components	Hilgenfeld et al. (1992)
Beta-Lactamase	MIR space station	Produced by bacteria and providing multi-resistance to β lactam antibiotics	Stoddard et al. (1992)
Human Interferon-γ	STS-26, STS-28	Antiviral agent, enhance immune cells activity	Ealick et al. (1991)
Bovine pancreatic trypsin inhibitor mutant	Russian unnamed re-entry capsule	Competitive inhibitor of several serine proteases as for example factor XIIa	Henning et al. (1994)

(continued)

Table 4.1 (continued)

Protein	Space Mission	Function/Target	Reference
HIV-1 reverse transcriptase complexed to a monoclonal antibody fragment and to a 19/18 base-paired double stranded DNA helical fragment	STS-50	One of the key players in the mechanism of infection by HIV retrovirus	DeLucas et al. (1994)
Human 1713-hydroxysteroid dehydrogenase in complex with NADP	MIR space station	Significant target for breast and prostate cancer drug therapy	Zhu et al. (1995)
T3Ri insulin hexamer, complexed with 4-hydroxybenzamide, human and bovine insulin	STS-57, STS-60, STS-95	Diabetes	Smith et al. (1996), Borgstahl et al. (2001)
Acidic phospholipase A_2	Chinese space mission, re-entry capsule	Inhibition of platelet aggregation, hemolysis, presynaptic and muscular toxicity	Pan et al. (1996)
Human Interferon α-2	STS-60	Hairy cell leukemia, multiple myeloma, venereal wart, AIDS related Kaposi's, sarcoma and chronic hepatitis B and C	Long et al. (1997)
Human Antithrombin	STS-67	Inactivation of several enzymes within the blood system	Wardell et al. (1997), Skinner et al. (1997)
Thaumatin	STS-73	Thaumatin production is induced in response to an attack of viroid pathogens	Ng et al. (1997)
STMV (Satellite tobacco mosaic virus)	STS-42	Plant Virus	Larson et al. (1998)
Collagenase	STS-65, STS-57	Only enzyme able to cleave native collagen under physiological conditions	Broutin et al. (1997), Broutin-L'Hermite et al. (2000)
Respiratory syncytial virus (RSV) antibody	STS-85	Human respiratory syncytial virus (HRSV) causes infections in the respiratory tract	Carter et al. (1999b)
HIV protease complex with cyclic inhibitor	STS-85	Vital role in HIV replication, HIV proteases are the main target in HIV drug therapies	Carter et al. (1999a, b)
EF-hand parvalbumin	STS-83	Cell excitation and relaxation, in muscles and in neurons	Declercq et al. (1999)

Table 4.1 (continued)

Protein	Space Mission	Function/Target	Reference
Herpes Simplex Virus 1 Single-Stranded DNA Binding Protein	STS-95	Herpes Simplex Virus 1 is responsible for most human cold scores	Mapelli and Tucker (1999)
Chaperonin-60	STS-84, JAXA	Assists in protein folding in mitochondria and plays a key function in autoimmune diseases	Kitano et al. (2000)
Thermus flavus Ribosomal 5S rRNA	STS-94	Involved in protein synthesis	Lorenz et al. (2000)
Alcohol Dehydrogenase from the Hyperthermophilic Archaeon *Sulfolobus solfataricus*	STS-73	Reversible interconversion of alcohols to aldehydes/ketones	Esposito et al. (2002)
Collagen triple helix model [(Pro-Pro-Gly)10]3	STS-95	Main component of connective tissue, the most abundant protein in mammals	Berisio et al. (2002)
NH3-dependent NAD+ synthetase from *Bacillus subtilis*	STS-95	Drugs designed special for bacterial NAD+ synthetases should decrease NAD+ levels in pathogens, thereby inhibiting bacterial growth	Symersky et al. (2002)
Human Bence-Jones dimer	STS-95	Amyloidogenic protein, light chains have the capacity to pass through the blood vessel endothelia and be converted into lethal, insoluble fibrils in vital organs like the kidney, heart, tongue, brain and skin	Terzyan et al. (2003), Alvarado et al. (2001)
Mistletoe lectin I from *Viscum album* in complex with adenine monophosphate	ISS-6A, STS-113	Ribosome-inactivating protein of type II, depurinates specifically ribosomal 23S/28S rRNA, causing inactivation of protein biosynthesis in eukaryotic cells, used in cancer therapy	Krauspenhaar et al. (2002)
Antibacterial peptide LC1	Chinese re-entry capsule Shenzhou-3	Antimicrobial function	Han et al. (2004)

(continued)

Table 4.1 (continued)

Protein	Space Mission	Function/Target	Reference
Human triosephosphate isomerase	ISS	Essential for human beings. It was inhibited by drug candidates, severe toxicity would arise	Kinoshita et al. (2005)
Hematopoietic prostaglandin (PG) D synthase	JAXA, STS-84	Therapeutic target enzyme for allergy and inflammation, mediates allergic and inflammatory reactions	Tanaka et al. (2011)
Hydrogenase maturation factor Hyp-F N-terminal	ISS-8A, STS-110	Hydrogen metabolism	Ponassi et al. (2011)
Mouse Lipocalin-Type Prostaglandin D Synthase	ISS, JAXA	Catalyzes the isomerization of PGH2, a common precursor of various prostanoids, to produce PGD2 and is involved in the regulation of pain	Inaka et al. (2011)
Phosphopantetheine Adenylyltransferase from *Mycobacterium Tuberculosis* in the Apo Form	ISS, Russian module	Involved in coenzyme A biosynthesis by catalyzing the penultimate and fourth step of this process	Timofeev et al. (2012b)
EGFR kinase domain (G719S/T790M) in the apo form	ISS	The human epidermal growth factor receptor (EGFR) is a multidomain protein and cancer drug target	Yoshikawa et al. (2013)
Thermostable T1 Lipase	ISS, JAXA	Catalyze both the hydrolysis of triglycerides and the synthesis of esters formed from alcohol and long chain fatty acids	Mohamad Aris et al. (2014)
Phosphoribosyl Pyrophosphate Synthetase from *E. Coli*	ISS, Russian module	Links the pentose phosphate pathway to the biosynthesis pathway of purine and pyrimidine nucleosides	Timofeev et al. (2016)
SaThiM ((*Staphylococcus aureus* Thiamin, 5-(hydroxyethyl)-4-methylthiazole kinase))	Chinese space mission Shenzhou-8	A potential target for pro-drug compounds	Drebes et al. (2016)

native drug target proteins, to obtain initial or more detailed understanding and higher resolution insights about the structure-function-relationship of the target biomolecule, and experiments to grow crystals of complexes with selected ligands or inhibitors. Also, most of such experiments were accompanied by methodical

experiments to obtain deeper and more thorough understanding about the fundamental phenomena within the crystallization processes and to transfer and utilize knowledge and information archived in space to support the optimization crystallization experiments on Earth.

Today it can be concluded, that space grown crystals did accelerate drug-discovery investigations (DeLucas et al. 1999), even initiated a drug discovery process in particular cases and also resulted in a number of spin-off companies utilizing such data for distinct drug design projects. Examples for the last are the pioneering experiments of Volker Erdmann and co-workers at the Free University Berlin, Germany, analyzing natural and modified nucleic acids (Vallazza et al. 2002, 2004) acting as antisense, microRNA, aptamers and ribozymes. In context of these investigations Erdmann invented and applied chemical modifications to stabilize nucleic acids by introducing for example LNA units, also known as "locked" nucleic acids.

As nucleic acids in comparison to proteins are known to be rather flexible and much more difficult to crystallize than proteins (Ducruix and Giege 1999), most RNA crystals obtained at this time showed rather limited diffraction power, as for example crystals of the *Thermus flavu*s ribosomal 5S RNA (Funari et al. 2000; Lorenz et al. 2000). In microgravity crystallization experiments the group of Erdmann and co-workers obtained high quality crystals of native and modified RNA molecules which were further used to collect diffraction data to high resolution. Structures solved and refined provided first and unique structural insights about non Watson Crick base pairs and involvement of solvent molecules stabilizing the phosphate backbone of these nucleotides as well as stabilizing none ideal base pairing and loops. Also the first structure of an entire LNA double helix (Fig. 4.1) and the first crystal structure of an RNA Racemate could be solved applying microgravity grown crystals (Vallazza et al. 2004; Rypniewski et al. 2006).

Between 1980 and 2009 the group of Erdmann performed seventeen crystallization experiments on different orbiters, five on satellite missions, ten on space shuttle missions and two long term experiments on the ISS. Experiments and results published and data obtained from these space experiments inspired and supported also the start up of three companies in Berlin, NOXXON GmbH, RiNA GmbH and Erdmann Technologies all focused on RNA biotechnology and application of distinct RNA aptamers and spiegelmers in drug design.

Beside German research activities utilizing microgravity crystallization in the field of RNA technologies and drug design, other investigations related to RNA research targeted structure-function-analysis of ribosome inactivating proteins (RIPs) type II and their particular structural features in cell recognition. Out of the family of RIPs only the heterodimeric glycoprotein Misteltoe I (ML-I) from *Viscum album* has high specific activities in recognition and binding to mammalian cells via a reversible and specific way to bind complex carbohydrates. ML-I consists of a toxic A-chain, which depurinates highly specific the ribosomal 23S rRNA and causes a fast and efficient inactivation of protein biosynthesis in eukaryotic cells. The galactose specific B-chain of ML-I facilitates the endocytic uptake to

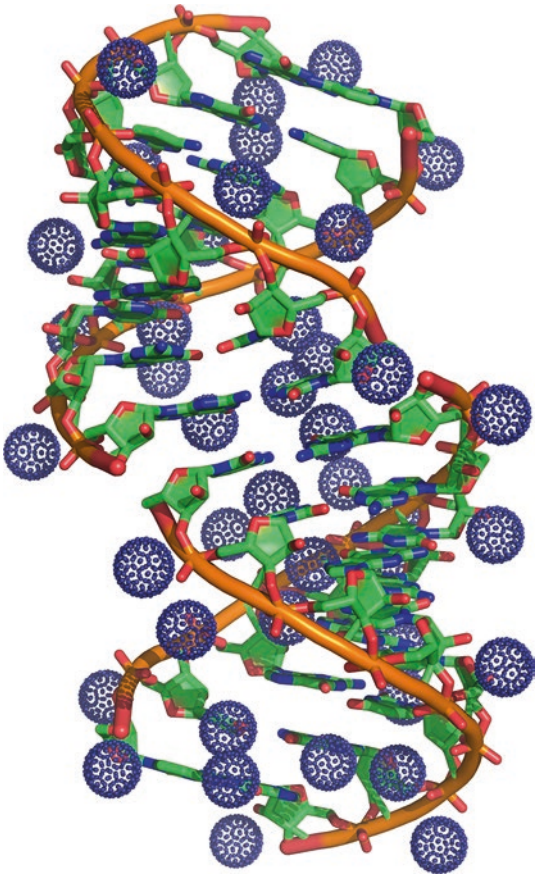

Fig. 4.1 RNA duplex in L-conformation (pdb code: 1R3O). Solvent waters stabilizing the structure are indicated by dashed spheres (Vallazza et al. 2004)

deliver the catalytically active A chain into the cytoplasm (Fig. 4.2). Therefore, ML-I is today a major component of therapeutically active substances applied in treatment of human cancer and cancer therapies (Hajto et al. 1989, 1990; Rostock and Huber 2004).

Due to the rather high crystal solvent content of approx. 75% and considering the glycosylation of the protein it is extremely difficult to obtain X-ray suitable ML-I crystals under lab conditions.

Crystals of ML-I obtained by vapor diffusion in microgravity, using the high-density protein crystal growth system (HDPCG) on the International Space Station (ISS) during mission ISS 6A (Krauspenhaar et al. 2002), showed significant better quality and diffraction data collected to 1.9 Å from those crystals allowed to analyze the active site conformation in complex with adenine, mimicking the RNA substrate. The high-resolution data revealed for the first time a N-glycosidase activity of the RIP type II protein, depurinating a single adenine in a high conserved rRNA GAGA-loop. Further microgravity crystallization experiments were performed to

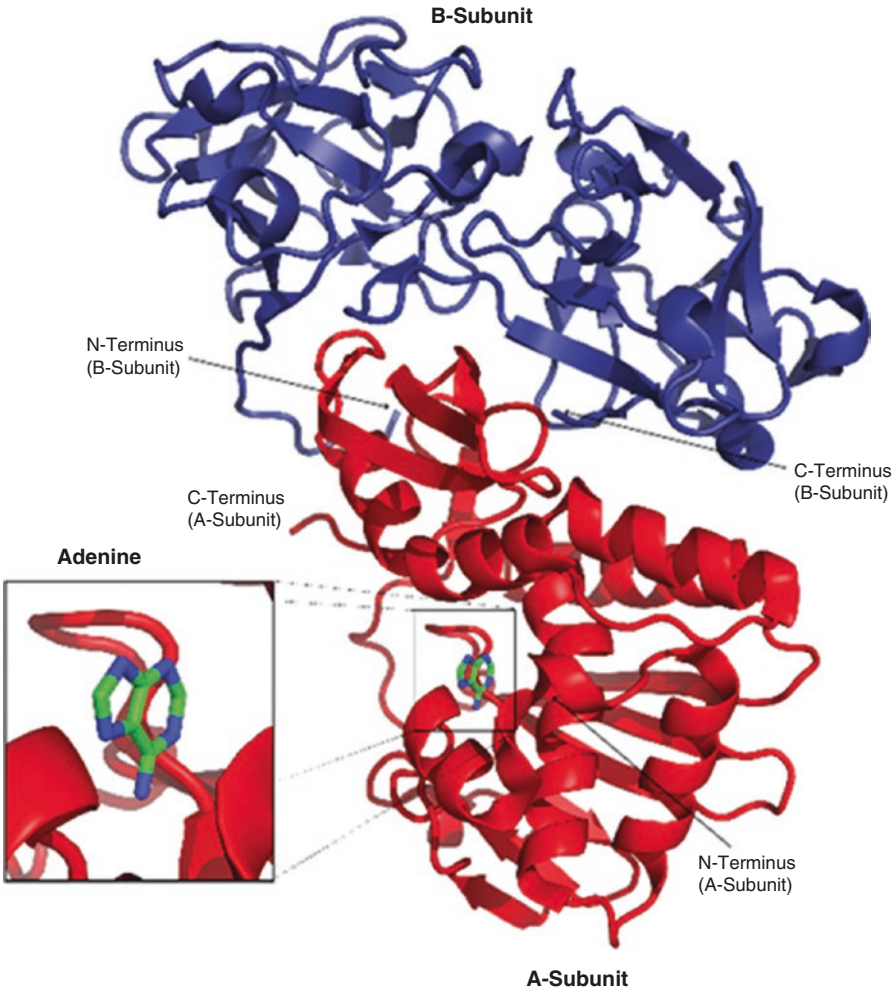

Fig. 4.2 Cartoon plot of Mistletoe Lectin I, in complex with Adenine (pdb code: 1M2T). Subunit B is shown on *top* in *blue* and subunit A, with the zoomed adenine binding cavity, below (Krauspenhaar et al. 2002)

obtain also more and detailed insights about the galactose-specific lectin activity of the B-chain and its specific functional features, as the B-chain is capable to bind most specific to cell surface receptors and triggers the endocytotic uptake of the toxic ML-I A-chain into cells (Krauspenhaar et al. 2002).

A group of German and Belgian scientists conducted in 1999 and 2001 pioneering crystallization experiments of surface-layer (S-layer) proteins from selected thermostable bacteria, applying the APCF system during shuttle flights STS-105 and STS-101 (Evrard et al. 1999; Claus et al. 2001). Up to this time, no X-ray suitable crystals

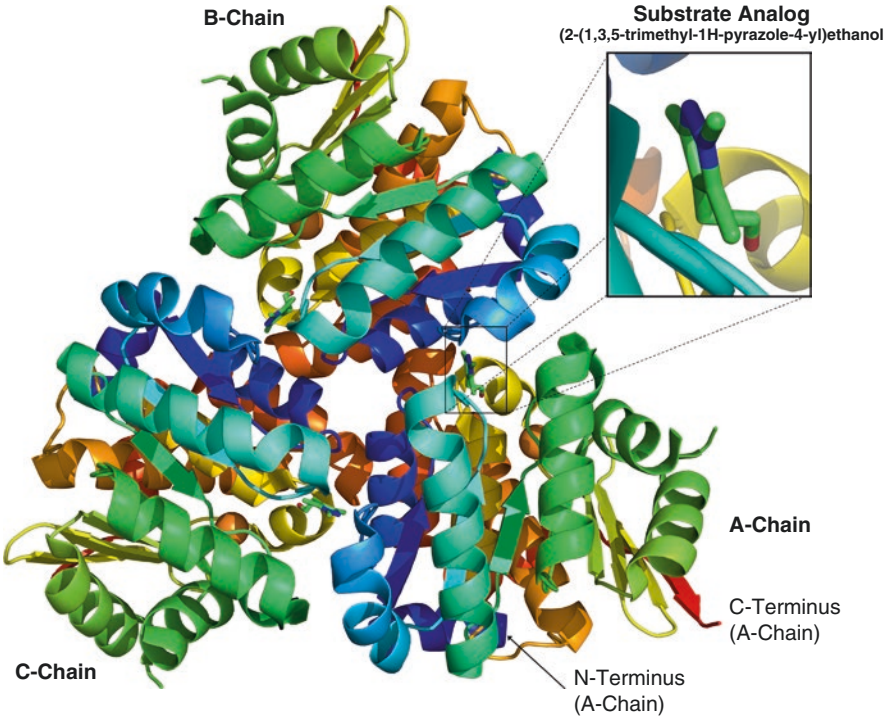

Fig. 4.3 Cartoon plot of the *Sa*ThiM trimer (pdb code: 5CGA). A, B and C chain are indicated. The active site regions are located in the interface regions of the subunits. One active site region in the interface between two monomers is indicated with bound substrate analog (Drebes et al. 2016)

of S-layer proteins were obtained. These types of proteins built and enclose via self-assembly entire cell surfaces of archaea and many other types of bacteria, showing high structural flexibility and relative low sequence homology between species (Büttner et al. 2015). As S-layer proteins are essential proteins in biofilm formation, they are today a major target in structural drug discovery investigations and several groups are working towards crystallization experiments to obtain X-ray suitable crystals.

In terms of a pro-drug approach to treat the multidrug and methicillin resistant bacteria *Staphylococcus aureus* (MRSA) crystals of a key enzyme in the vitamin B1 biosynthetic pathway, a 5-(hydroxyethyl)-4-methylthiazole kinase (*Sa*ThiM; EC 2.7.1.50), were grown on the Chinese Shenzhou-8 mission in terms of a cooperative project between the Chinese and German space organizations. Earth grown crystals of the enzyme ThiM showed throughout an unfortunate structural heterogeneity and internal pseudo-symmetry not allowing to obtain high resolution data of complexes with selected pro-drug compounds. Space grown crystals provided the basis to collect X-ray data to high resolution with three selected lead compounds (Fig. 4.3); the results were recently published (Drebes et al. 2016).

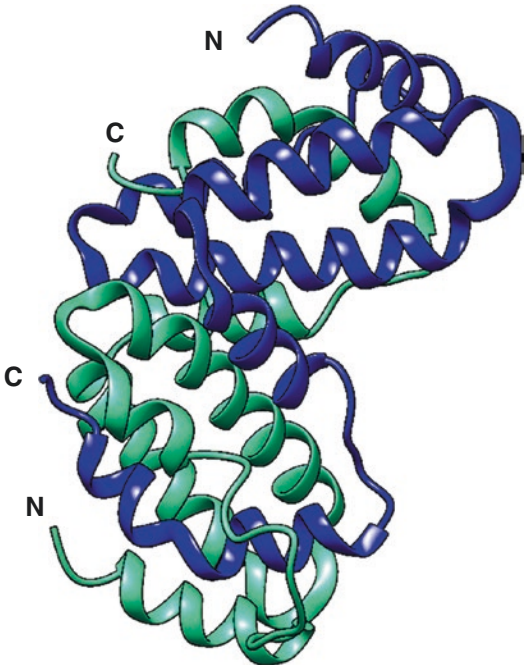

Fig. 4.4 Cartoon plot of a human γ interferon dimer (pdb code: 1HIG). A and B chain are indicated in *blue* and *green*, respectively (Ealick et al. 1991)

Further microgravity crystallization experiments successfully performed by international research collaborations in terms of drug discovery investigations and worth to be highlighted are: Experiments performed during the space shuttle mission STS-26 by Ealick and co-workers (Ealick et al. 1991; DeLucas et al. 1999) provided crystals of human γ interferon (Fig. 4.4), allowing to solve and analyze the structure-function-relationship of the protein exhibiting pleiotropic biological functions.

γ interferon was first applied as antiviral agent, and later recognized to enhance also the immune cell activity and being involved in several immune regulating functions (Ealick et al. 1991). To understand different and distinct oligomeric conformation of human and bovine insulins microgravity crystallization experiments were performed on several STS missions (Long et al. 1997; Smith et al. 1996, 2003). Structural data also published in collaboration with the pharma company Lilly highlight substantial improvements of insulin crystals grown in space, allowing a more detailed structure analysis (Borgstahl et al. 2001).

In terms of inhibitor and drug design studies a collagenase from *Hypoderma lineatum* was crystallized during the IML-2 Spacelab mission and analyzed in collaboration with scientists from the company Gallaxo (Broutin-L'Hermite et al. 2000). Structural data (Fig. 4.5) at 1.7 Å resolution allowed to identify enzyme-inhibitor specific features.

In context to this topic an extended collagen-like polypeptide was crystallized by Zagari and co-workers (Berisio et al. 2000, 2002), applying a dialysis technique during the space shuttle mission STS-95. Data reported describe significantly better

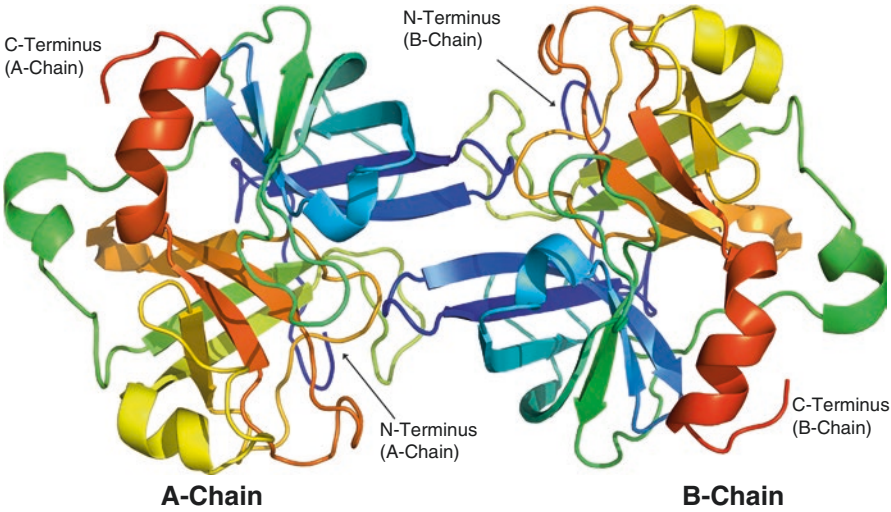

Fig. 4.5 Cartoon plot of the dimeric HL Collagenase (Broutin-L'Hermite et al. 2000)

diffracting crystals than those grown on Earth, allowing diffraction data collection to 1.3 Å resolution.

Another example of drug design investigations is the crystallization of the membrane associated human estrogenic 17 β-hydroxysteroid dehydrogenase, a drug target to treat breast and prostate cancer. The human dehydrogenase was crystallized aboard the Russian MIR space station in 1994 by Lin and co-workers from the University of Quebec (Zhu et al. 1995). The authors reported crystals allowing higher resolution diffraction data collection, compared to Earth grown crystals. The same group performed in 2002 in collaboration with scientist from the Academy of Sciences in China further crystallization experiment on the Chinese Shenzhou-3 flight (Han et al. 2004). In total, five different proteins with pharmaceutical relevance were crystallized on board Shenhou-3, and out of these, crystals of four proteins were obtained with significant higher quality compared to ground controls. Interesting is, that one antibacterial peptide yielded X-ray suitable crystals only in microgravity, which diffracted to 2.0 Å resolution (Han et al. 2004).

Bi and co-workers from the Academy of Sciences in Beijing (Pan et al. 1996) analyzed a snake venom phospholipase A_2 to 1.8 Å resolution applying crystals grown on a Chinese satellite mission in 1994. For these experiments, improved crystal quality was confirmed and approved by comparing in detail relative Wilson plot statistics. Venom PLA_2 enzymes are involved in platelet aggregation, hemolysis and muscular toxicity. The authors used structural data obtained for the design of specific PLA_2 inhibitors.

The research collaboration around D. Smith of the Hauptman-Woodward Medical Research Institute report the crystallization of different insulins and Human Interferon α-2 during the shuttle missions STS-51 and STS-52 utilizing the PCF hardware and applying a macroscale temperature approach to induce crystallization

(Long et al. 1997). IFNa2 is known and applied in antiviral and antitumor applications. The crystallization experiments had two objectives, first the production of high quality protein crystals for X-ray analysis and structure-based drug design, and second the preparation of large quantities of relatively contaminant free crystals for particular time-delayed and triggered drug delivery procedures.

Improved diffraction and better ordered crystals were reported for the glycoprotein antithrombin crystallized in microgravity (Wardell et al. 1997) applying the PCAM hardware, the protein crystallization apparatus for microgravity (Carter et al. 1999b). This experiment confirmed again that microgravity can support the production of even very difficult to grow proteins, like glycosylated proteins. Antithrombin is multifunctional and interacts with several enzymes of the blood coagulation system.

The nucleosome core particle (NCP), the fundamental building block of chromatin was crystallized by Bunik and co-workers (Harp et al. 2000), applying a modified version of PCAM, the diffusion controlled crystallization apparatus (DCAM) during the USML2 shuttle mission (Carter et al. 1999a). Crystals obtained allowed first time data collection to 2.5 Å resolution, in comparison to ground grown crystals which showed high anisotropy in the diffraction pattern (Fig. 4.6).

A further crystallization experiment worthwhile to mention is the crystallization and following structure analysis of urinary immunoglobulin light chains, also referred to as human Bence-Jones proteins (Bence-Jones 1848), having the ability

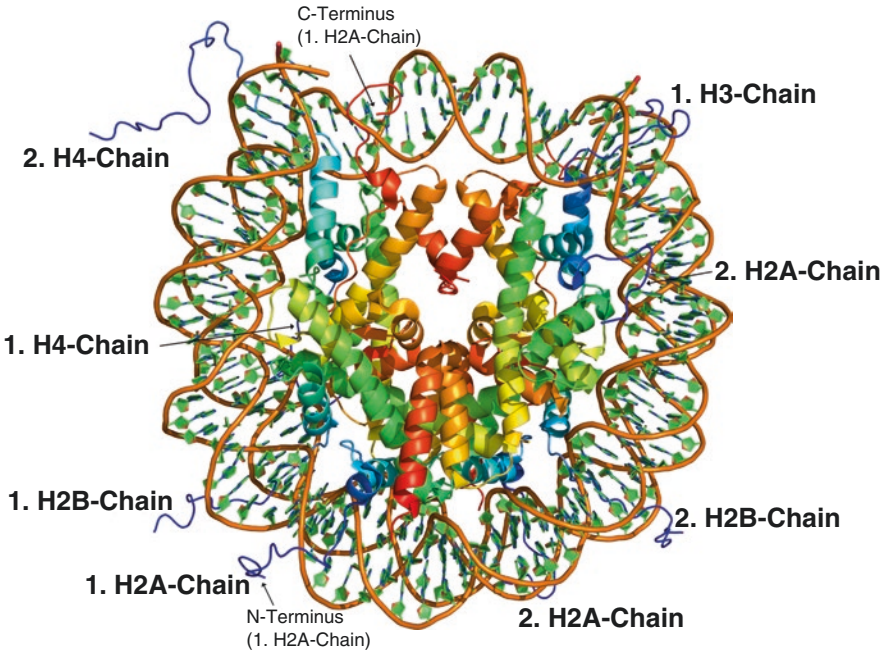

Fig. 4.6 Cartoon plot of the entire nucleosome core particle (pdb code: 1EQZ; Harp et al. 2000)

to first pass through the blood vessel endothelia and showing high structural flexibility to convert into lethal and insoluble fibrils in vital organs like the kidney, heart, tongue, brain and skin. The human Bence-Jones dimer applied for the experiments showed similar amyloidogenic properties in patients and *in-vitro* experiments. Edmundson and his group performed two microgravity experiments on the shuttle missions STS-80 and STS-95 applying vapor diffusion in distinct capillaries and reported improved crystals allowing a more conclusive interpretation of the tertiary structure and voluminous ligand binding regions. The microgravity crystallization experiment further indicated, that a more extended crystallization period could in principle yield crystals suitable for neutron diffraction studies (Alvarado et al. 2001; Terzyan et al. 2003). A Russian research collaboration performed several crystallization experiments between 2006 and 2012 on the Russian Module of the ISS focusing on drug discovery investigations. The scientists selected proteins of high pharmaceutical relevance like the phosphopantethene adenylyltransferase from *Mycobacterium Tuberculosis*. Unfortunate, only some data about these experiments were reported in international journals, as for example some translated articles published in the journal Crystallographic Reports. The articles summarize and describe predominantly final structural data, however indicate as well that crystals obtained in microgravity had high quality for X-ray data collection (Timofeev et al. 2010a, b, 2012a, 2013a, b, 2016).

Data and results summarized before having convincingly demonstrated that crystallization experiments under microgravity conditions have markedly contributed to the progress in drug discovery and design. In some cases even start-up companies have been successfully established. Crystallization experiments presently running on the ISS or upcoming in the near future will certainly stimulate further drug design investigations, considering also latest advantages in more routine sample transport to the ISS and crystal transport back to the labs of the principle investigators. Overall, the benefit in treating certain diseases is obvious.

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Chapter 5
Cell Biology in Space

Daniela Grimm

Abstract This chapter provides an overview of experiments conducted in space and on Earth using machines created to simulate microgravity. Today, research in space on the International Space Station (ISS) or in orbit, as well as the exploration by humans of extraterrestrial environments like the Moon or Mars, is of worldwide interest. The commercial use of space and future space tourism will further increase this interest. The space travels of European astronauts have contributed to this great success with their enormously positive PR activities before, during and after their respective missions.

In the past, space medicine and gravitational biology were disciplines familiar only to a small research community, but they are attracting a lot of interest today. A large number of exciting research findings have been discovered in the last 40 years. Today we know that microgravity has an enormous influence on the biology of human cells, in particular on cellular morphology, the cytoskeleton and growth behavior. Moreover, it changes various biological processes in human cells.

Keywords Microgravity • Human Cells • Cytoskeleton • Multicellular Spheroids • Extracellular Matrix

5.1 Introduction

Analysis of the cellular response to real microgravity in space offers new aspects in cell biology, tissue engineering and cancer research. Studies of the cellular response to microgravity have revealed novel adaptive mechanisms. Growing cells in a microgravity environment induces a three-dimensional (3D) growth behavior in different cell types more closely representing the *in vivo* situation in the human body (Grimm et al. 2014). This chapter summarizes data regarding space experiments conducted aboard the space shuttle, *MIR*, and the International Space Station (ISS). In addition, experiments obtained aboard unmanned space missions (SIMBOX/Shenzhou-8), rocket missions or parabolic flight missions will be discussed (Fig. 5.1). To compare and validate these findings, experiments using so-called ESA ground-based facilities,

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Fig. 5.1 Platforms to provide real microgravity. (**a**) Shenzhou-8 rocket; (**b**) Parabolic Flight Plane Airbus A300; (**c**) TEXUS rocket launch at ESRANGE, Kiruna, Swedens

such as the Random Positioning Machine (RPM), the 2D clinostat and the NASA-developed Rotating Wall Vessel (RWV) bioreactor, will be evaluated (Fig. 5.2).

Long-term space missions induce a variety of health problems in astronauts, cosmonauts and taikonauts (White and Averner 2001; Grimm et al. 2011, 2016). Examples of these are bone loss, osteoporosis and cardiac atrophy together with hypotension and arrhythmias, muscle atrophy or a dysfunction of the immune system (White and Averner 2001; Grimm et al. 2011, 2016). In addition, visual problems are considered to be a major complication of spaceflights. One relevant health concern is the dysfunction of the immune system. This can result in opportunistic infections, or a poor wound-healing process.

One of the main aims of the current research on space medicine is to evaluate the effects of microgravity on human cells. Therefore, investigations of the primary molecular mechanisms of how microgravity might affect cell signaling are currently of interest.

5.2 Human Adult Retinal Pigment Epithelium Cells

A long-term stay in orbit can affect the eyes and might result in visual impairment for space travellers. Identification of the underlying mechanisms is very important. Studies of astronauts revealed spaceflight-induced ocular changes such as choroidal folds, optic disk edema, globe flattening and hyperopic shifts (Mader et al. 2011). It has been hypothesized that these visual problems are connected to cephalad fluid

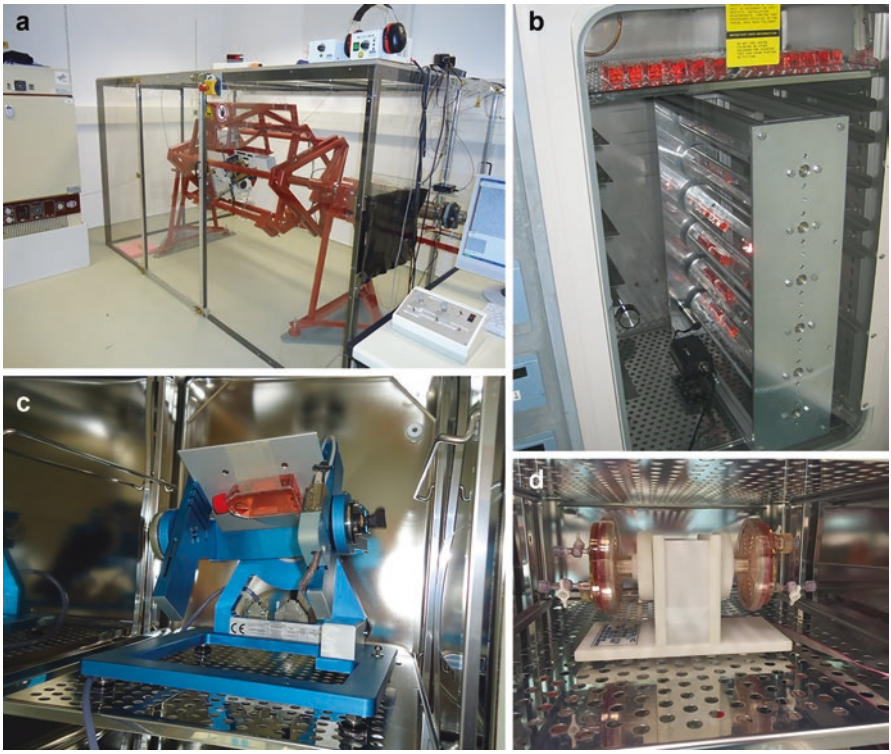

Fig. 5.2 ESA ground-based facilities: (**a**) 2D clinostat microscope for the observation of samples during fast rotation around one axis perpendicular to gravity (Group PD Dr. Ruth Hemmersbach, Gravitational Biology, DLR Cologne, Germany); (**b**) Fast rotating 2D clinostat in an incubator with samples (constructed by PD Dr. Ruth Hemmersbach's group, Gravitational Biology, DLR Cologne); (**c**) Desktop Random Positioning Machine (Airbus, Defense & Space; former Fokker Space, Leiden, NL); and (**d**) Rotating Wall Vessel in an incubator with a chondrocyte experiment (Synthecon Inc., Houston, TX, USA, delivered by Cellon SA, Bascharage, 4940 Schouweiler, Luxembourg)

shifts, intracranial pressure and optic nerve sheath compartment syndrome, as a consequence of longterm microgravity exposure (Zwart et al. 2016; Mader et al. 2016).

The risk of visual impairment is an important health concern for NASA. We had recently examined the effects of simulated microgravity on human adult retinal epithelium cells (ARPE-19 cells). This study showed alterations in the cytoskeleton of ARPE-19 cells (Fig. 5.3). This was paralleled by changes in cell growth and the expression patterns of selected genes involved in cell structure, shape, adhesion, extracellular matrix, migration and angiogenesis (Corydon et al. 2016a).

5.3 Lymphocytes Cultured Under Conditions of Microgravity

A dysfunction of the immune response of astronauts is already evident after a few days in space and after longterm space flights (Pietsch et al. 2011).

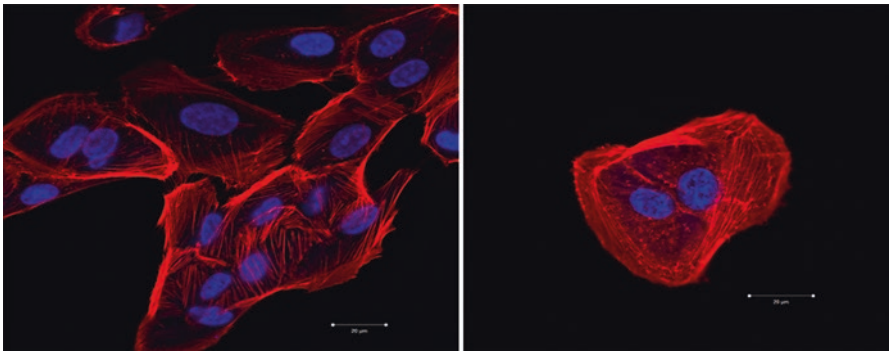

Fig. 5.3 Confocal laser scanning microscopy of rhodamine-phalloidin-stained ARPE-19 cells grown under conditions of 1*g* and after RPM-exposure. Blue staining: DAPI (4′,6-diamidino-2-phenylindole) highlights the nucleus; red staining: rhodamine-phalloidin to visualize the F-actin

Therefore, many researchers have demonstrated in the course of nearly 40 years of space research that human cells subjected to microgravity reveal a number of alterations in structure and function, including changes in proliferation, but also changes in cytokine production, or in protein kinase C distribution, as well as an elevation of programmed cell death.

To find the reasons for the altered immune response, researchers examined isolated lymphocytes during the first Spacelab missions and focused on the proliferation of these cells (Cogoli et al. 1979). In other studies, they detected an impairment of the response of lymphocytes to mitogenic stimulation (Cogoli et al. 1984; Cogoli and Tschopp 1985).

In addition, signal transduction processes for T-cell activation were disturbed (Cogoli-Greuter 1998; Cogoli-Greuter et al. 2004). In parallel, microgravity induced an increase of apoptosis and enhanced apoptosis-associated Fas/APO-1 proteins in lymphocytes (Jurkat cells) (Lewis et al. 1998).

Maccarrone et al. (2003) demonstrated the induction of 5-lipoxygenase activity and cytochrome c release into the cytosol of human lymphocytes, a result obtained under simulated microgravity conditions and later proven in space on the ISS (Battista et al. 2012). In addition, apoptosis was accompanied by an imbalance of interleukin-2 (IL-2) and interferon-γ (INF-γ), as well as anti- and proapoptotic cytokines (Gasperi et al. 2014). 5-LOX inhibition reduced apoptotic death, restored the initial IL-2/INF-γ ratio and reverted μ-calpain activation induced by simulated microgravity (Gasperi et al. 2014).

In space and on Earth the activation of human T lymphocytes is reduced. Furthermore, human leukocytes exerted changes in the protein kinase C (PKC) distribution (Hatton et al. 1999) and in the IL-2 and IL-2-R-alpha expression (Galleri et al. 2002). In addition, protein kinase A (PKA) is involved in sensing gravity (Boonyaratanakornkit et al. 2005).

In the spaceflight experiment LEUKIN, the investigators found that the transcription of immediate early genes is inhibited in T cells activated in microgravity and that disrupted activation of Rel/NF-κB, CREB1 and SRF transcription factors is involved (Chang et al. 2012).

It had been demonstrated that human T lymphocytes showed a differential inhibition of transcription factor activation in modelled microgravity created by clinorotation (Morrow 2006). AP-1 activation was blocked by clinorotation, whereas NFAT dephosphorylation occurred. Clinorotation inhibits the activation of cellular signaling (Morrow 2006). Another study investigating non-activated human T lymphocytes during a parabolic flight mission showed a downregulation of CD3 and IL-2R surface receptor after 20 s (Tauber et al. 2015). The authors assume that a gravity condition of 1*g* is required for the expression of key surface receptors and appropriate regulation of signal molecules in T lymphocytes (Tauber et al. 2015). These data show that several transcription factors play a role in sensing gravity in human lymphocytes.

Adrian et al. (2013) investigated NR8383 rat alveolar macrophages under altered gravity conditions obtained by parabolic flight maneuvers and clinorotation (2D–clinostat) and focused on the oxidative burst reaction in macrophages, which is a key element in the innate immune response and cellular signaling processes. Their data showed that gravity-sensitive steps are located both in the first activation pathways and in the final oxidative burst reaction. This could be explained by the role of cytoskeletal dynamics in the assembly and function of the NADPH oxidase complex (Adrian et al. 2013).

In CD3/CD28-stimulated primary human T cells, the p21 mRNA expression increased 4.1-fold after 20 s in real microgravity during a parabola in primary CD4+ T cells and 2.9-fold in Jurkat T cells, compared with 1g in-flight controls after CD3/CD28 stimulation. The histone acetyltransferase (HAT) inhibitor curcumin was able to abrogate microgravity-induced p21 mRNA expression, whereas its expression was enhanced by a histone deacetylase (HDAC) inhibitor. The authors supposed that cell cycle progression in human T lymphocytes requires Earth gravity and that the disturbed expression of cell cycle regulatory proteins could contribute to the downregulated immune system of humans in space (Thiel et al. 2012).

5.4 Vascular Cells in Space

Endothelial cells are important for the integrity of the vascular wall. They form the inner layer of blood vessels throughout the whole organism and serve as an anticoagulant barrier between blood and the vessel wall. It is a unique multifunctional cell with important basal and inducible metabolic and synthetic functions (Infanger et al. 2006). It is known that modulation of the endothelial cell function can induce cardiovascular problems and other health problems of humans in space. Endothelial cells are important for several biological processes, such as immune regulation, blood coagulation, growth, extracellular matrix synthesis and others. These processes can be disturbed when the cells are exposed to altered gravity conditions.

Endothelial dysfunction of microvascular endothelial cells (MVECs) may contribute to cardiovascular deconditioning occurring in microgravity. Microgravity conditions induced apoptosis in MVECs. The authors found a downregulation of the PI3K/Akt pathway, an elevation of NF-κB and a depolymerization of F-actin (Kang et al. 2011). Moreover, simulated microgravity can trigger angiogenesis in

endothelial cells and induce tube and spheroid formation (Grimm et al. 2009, 2010, 2014; Ma et al. 2014a, b; Aleshcheva et al. 2016).

A recent paper by Shi et al. showed that clinorotation induces eNOS-mediated angiogenesis in HUVEC cells (Shi et al. 2016). In addition, the authors showed a requirement for Cav-1-associated signaling in microgravity-driven angiogenesis. They suggest that Cav-1 is a critical mediator in simulated weightlessness in endothelium-dependent angiogenesis (Shi et al. 2016).

It has been shown that both P2 receptor gene and protein expression in endothelial cells were altered under clinostat exposure. This indicates that P2 receptors might be important players responding to gravity changes in vascular cells (Zhang et al. 2014).

The post-flight microarray analysis of the ISS SPHINX experiment (HUVEC cells in space) revealed 1023 significantly modulated genes (Versari et al. 2013). The thioredoxin-interacting protein was 33-fold increased, and heat-shock proteins 70 and 90 5.6-fold downregulated. Ion channels, mitochondrial oxidative phosphorylation and focal adhesion were widely affected (Versari et al. 2013). The SPHINX investigators demonstrated that the space environment influences signaling pathways, inducing inflammatory responses, changing endothelial behavior and promoting senescence (Versari et al. 2013).

Endothelial cells exposed to short episodes of real microgravity achieved during parabolic flights exhibited changes in the cytoskeleton and a differential gene expression (Grosse et al. 2012a). In addition, this work showed that caveolins, AMPKα1 and integrins are possible gravi-sensitive elements (Grosse et al. 2012a).

Taking the available results together, protein kinases, integrins, caveolin-1, eNOS, P2 receptors and NF-kB might be key players in sensing gravity in endothelial cells.

5.5 Chondrocytes and Bone Cells

A prolonged exposure to microgravity has deleterious effects on human bone and cartilage (Grimm et al. 2016). Crewmembers suffer after a long-term spaceflight from a reduction of cartilage mass due to mechanical unloading (Zayzafoon et al. 2005).

Neocartilage was formed by porcine chondrocytes cultured in microgravity during the spaceflight 7S (Cervantes mission) on the ISS (Stamenkovic et al. 2010). A weaker extracellular matrix staining of ISS neocartilage tissue was detectable. Higher collagen II/I expression ratios were observed in ISS samples than in control tissue. In addition, there was a lower cell density in ISS neocartilage, which was significantly reduced compared with the normal-gravity neocartilage tissues.

Recent results from ten astronauts who spent more than 5 months in space showed that the cartilage extracellular matrix is sensitive to prolonged exposure to microgravity. This is supported by changes in serum molecular biomarker levels of cartilage turnover. A reduced mechanical loading through microgravity seems to initiate catabolic processes (Niehoff et al. 2016).

Shortterm microgravity during parabolic flight maneuvers had no damaging effects on human chondrocytes. The viability of the cells was normal during the parabolic flight, and a clearly elevated expression of anti-apoptotic genes was detectable after 31st parabolas (Wehland et al. 2015).

A similar result was obtained when human chondrocytes were cultured on the random positioning machine. No signs of apoptosis could be detected (Ulbrich et al. 2010).

Shortterm studies of human chondrocytes exposed to the RPM demonstrated early cytoskeletal changes within 30 min (Aleshcheva et al. 2013). No cytoskeletal changes in chondrocytes were detectable after the first parabola, but they appeared later (vimentin, tubulin, cytokeratin) after the 31st parabola of a parabolic flight, although F-actin remained unaltered (Aleshcheva et al. 2015).

Taking these results for chondrocytes together, chondrocytes exposed to microgravity exhibit only moderate changes of the cytoskeleton. After RPM exposure they change their extracellular matrix production behaviour while they rearrange their cytoskeletal proteins prior to forming three-dimensional aggregates (Fig. 5.4). No signs of programmed cell death were detectable.

A longterm stay in space without available countermeasures enormously affects the bone health of astronauts (Grimm et al. 2016). Bone loss, osteoporosis and bone fractures can occur. One of the mechanisms through which space ventures depress bone formation is through effects on the Wnt/β-catenin signaling pathway. Recent studies have shown that simulated microgravity conditions increase the expression of sclerostin and Dkk-1 in osteocytes (Yang et al. 2015). Wnt-signaling elevates osteoblastic cell differentiation and bone formation, but also inhibits bone resorption. It induces blocking of the receptor activator of nuclear factor-κB-ligand (RANKL)/RANK interaction (Jackson et al. 2005).

There is evidence that although the exact mechanisms are not known, it is possible that sclerostin plays a key role in skeletal adaptation to mechanical forces. Spatz

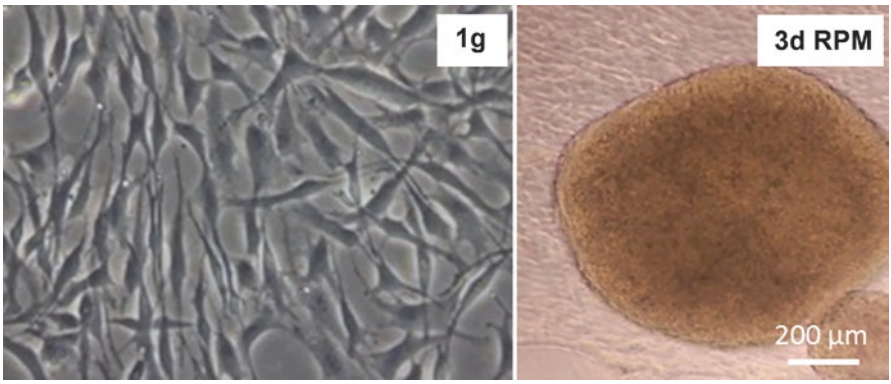

Fig. 5.4 Human chondrocytes (Provitro, Berlin, Germany) cultured under 1*g*–control conditions grew as a 2D monolayer (*left picture*). When they were exposed to the RPM they grew in the form of 3D aggregates and as a 2D monolayer (*right picture*). Phase contrast microscopy was performed by using a Leica microscope (Microsystems GmbH, Wetzlar, Germany). Pictures were taken with a Canon EOS 550D (Canon GmbH, Krefeld, Germany)

et al. investigated the expression of sclerostin in osteocytes *in vitro* and showed that sclerostin is upregulated by mechanical unloading (Spatz et al. 2015). This suggests that mechanical loading regulates intrinsic osteocyte responses (Spatz et al. 2015).

A 5-day spaceflight resulted in an increase in bone resorption by osteoclasts as well as a decrease in osteoblast cellular integrity (Nabavi et al. 2011). Osteoblasts exposed to microgravity exhibited alterations of the microtubules, changes in focal adhesions, and thinner cortical actin and stress fibres (Nabavi et al. 2011).

Real microgravity induces alterations of the cytoskeleton and focal adhesions in bone cells. These are two major mechanosensitive structures. The cytoskeleton responds to changes in the mechanical environment because it is connected to the extracellular matrix through focal adhesions. Exposure of osteoblasts to microgravity impairs their cytoskeleton stability and reduces cellular tension, as well as focal adhesion formation and stability (Hughes-Fulford 2003). Kumei et al. demonstrated that microgravity influences cell adhesion, the cytoskeleton and extracellular matrix proteins of rat osteoblasts cultured in space. Osteopontin and tubulin gene expression levels were downregulated (Kumei et al. 2006).

After a 48 h-clinostat exposure, actin filaments of human osteosarcoma MG63 cells depolymerized, became thinner, and showed a dispersed distribution and disorder, especially in the cytoplasm (Dai et al. 2013).

Summarizing these findings on bone, microgravity clearly influences the cytoskeleton and extracellular matrix proteins in bone cells.

5.6 Cancer Cells Cultured in Microgravity

Different types of cancer cells exposed to microgravity exhibited specific alterations of the cytoskeleton. Human breast cancer cells exposed to real microgravity conditions in space revealed alterations in their microtubules as well as changes in the perinuclear cytokeratin network (Vassy et al. 2001).

These cytoskeletal changes are detectable early. After the first parabola during a parabolic flight, alterations of F-actin were found in follicular thyroid cancer cells (ML-1 cell line) (Ulbrich et al. 2011) and also observed in endothelial cells (EAhy926 cell line) (Grosse et al. 2012a). These findings were proven by the FLUMIAS (Fluorescence Microscopy Analysis System) experiment under microgravity (Fig. 5.5). During the TEXUS-52 sounding rocket flight, the FLUMIAS microscope revealed similar changes in the F-actin network to those observed after RPM-exposure (Corydon et al. 2016b).

When cancer cells were cultured under microgravity conditions, they started to grow three-dimensionally within 24 h, depending on the cell type. DU-145 human prostate carcinoma cells were cultured on a HARV (high-aspect rotating-wall vessel). The authors found that HARV cultivation induced a 3D organoid-like growth type, which was less aggressive, slower growing, less proliferative, more differentiated and a less pliant cell method (Clejan et al. 2001). A similar finding that microgravity induces a phenotype switch to a less aggressive one was detected for

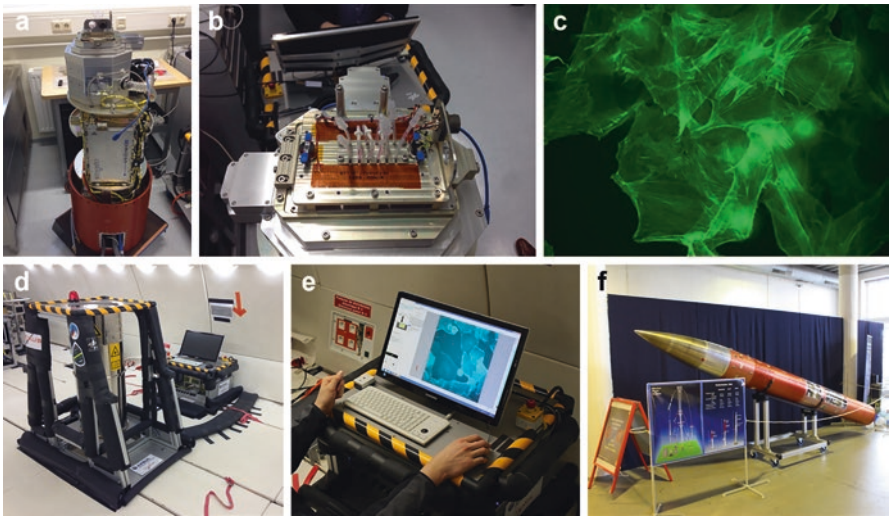

Fig. 5.5 (**a**) Confocal laser scanning microscope (CLSM); (**b**) cross table with observation unit, cells on a slide are fixed on it; (**c**) picture of transfected FTC-133 thyroid cells, the green fluorescence enables the observation of the cytoskeleton (F-Actin); (**d**) CLSM with housing for the testing during the 24th DLR parabolic flight campaign (02.2014); (**e**) picture of FTC-133 cells in the parabolic flight plane; (**f**) TEXUS rocket payload for exhibitions

low-differentiated follicular thyroid cancer cells, which were investigated during the Sino-German space mission Shenzhou-8 in real microgravity (Ma et al. 2014b).

Moreover, 3D growth and the formation of 3D spheroids were confirmed in space (Pietsch et al. 2013). A scaffold-free formation of extraordinarily large 3D aggregates by thyroid cancer cells with altered expression of EGF and CTGF genes was detectable in space. The formation of 3D spheroids had already been demonstrated by exposing a variety of cancer cells to simulated microgravity conditions using a NASA rotary cell culture system (Ingram et al. 1997). The expression of the cell adhesion molecules CD44 and E cadherin was upregulated in the 3D constructs (Ingram et al. 1997).

Follicular thyroid cancer cells investigated on the RPM started to grow in the form of two phenotypes: first as a 2D monolayer and second as a 3D spheroid within 24 h (Grimm et al. 2002; Grosse et al. 2012b; Warnke et al. 2014; Svejgaard et al. 2015; Kopp et al. 2015). Cells grown on the RPM for 24 h exhibited an increase in the NF-κB p65 protein and apoptosis compared to 1g controls, a result also found earlier in endothelial cells. The signaling elements IL-6, IL-8, OPN, TLN1 and CTGF are involved with NF-κB p65 in RPM-dependent thyroid carcinoma cell spheroid formation (Grosse et al. 2012b). In addition, a device comparison study (RPM and 2D clinostat) demonstrated changes in the regulation of *CTGF* and *CAV1* appearing in a comparable manner on both machines. Both factors seem to play a role in 3D formation (Warnke et al. 2014). IL-6 and IL-8 application to the medium of ML-1 cancer cells has shown a direct influence on 3D formation (Svejgaard et al.

2015). In a longterm study, FTC-133 low-differentiated follicular thyroid cancer cells and normal thyrocytes were cultured on the RPM for 14 days (Kopp et al. 2015). Significant differences between normal and cancer cells were found concerning the gene expression of *NGAL, VEGFA*, *OPN*, *IL6* and *IL17* and the secretion of VEGFA, IL-17 and IL-6 (Kopp et al. 2015). These data suggest their involvement in 3D formation of thyroid cells after RPM exposure.

Taking these data together, cancer cells studied in real or simulated microgravity show early cytoskeletal changes, apoptosis and 3D growth. Several factors are known to be involved in the aggregation process and phenotype switch of the investigated cells. These are the growth factors CTGF, EGF and VEGF, the cytokines IL-6, IL-8 and IL-17 and NF-kB.

5.7 Hypothesis on How Gravity Is Perceived by Human Cells: The Tensegrity Model—How Unspecialized Human Cells Might Sense Gravity

As described, human cells *in vitro* can react to mechanical unloading in different ways; however, the question arises as to how they are able to sense the rather weak changes in force.

Ever since Rijken et al. found significant alterations of the cytoskeleton in human A431 cells during a TEXUS flight in 1991 (Rijken et al. 1991a, b), the cytoskeleton has been a hot candidate for transmitting mechanical unloading from the cells' environment.

How the cells manage to transform the mechanical signal into a biochemical one is still today a current topic under investigation. However, an increasing yield of data supports the tensegrity model hypothesis, proposed by Ingber (1999).

The tensegrity model claims that cells are hardwired by the different parts of the cytoskeleton, which are connected to discrete cell adhesions. According to this model, cells are spanned open and are under continuous tension. The adhesion points are connected to the extracellular matrix. In sum, there is a balance of force between the extracellular matrix, adhesion points and the cytoskeleton in normal gravity conditions. Therefore, an imbalance of adhesion and cytoskeleton would result in a change of cell shape and have a direct impact on signaling cascades and downstream transcription events (Ingber 1999).

This theory is supported by various findings of cytoskeletal changes in different cell types after short-term exposure to real and simulated microgravity (Vorselen et al. 2014). Fixation of thyroid cancer cells and endothelial cells as well as chondrocytes after 22 s of microgravity revealed that actin fibres and/or microtubules were localized close to the nucleus while losing their distinct polarization (Grosse et al. 2012a; Aleshcheva et al. 2015; Ulbrich et al. 2011). These findings are in concert with significant gene expression changes after 22 s of real microgravity.

However, artefact building during fixation could not be neglected until Corydon et al. first investigated life-act GFP marked thyroid cancer cells during a parabolic

flight campaign and the TEXUS 52 campaign in Kiruna, Sweden (Corydon et al. 2016b). Live imaging of the cells during microgravity revealed an instant rearrangement of actin filaments and a rapid change of cell shape.

These findings are in concert with gene expression changes in cytoskeletal genes and proteins, which are involved in proliferation and differentiation. Finally, these experiments further increased the evidence of a direct correlation between the cytoskeletal rearrangements upon microgravity and transcription alterations and strongly suggest that the interaction between the extracellular matrix, adhesion and connected cytoskeleton is a major part in gravi-sensing non-specialized human cells.

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Chapter 6
Tissue Engineering in Microgravity

Markus Wehland and Daniela Grimm

Abstract Tissue engineering enables the development of functional constructs from cells and has different applications in regenerative medicine and drug screening but also in non-therapeutic approaches.

In the course of several space flight missions as well as ground-based experiments, it has been shown that both real and simulated microgravity can induce the formation of three-dimensional tissues in different human cell types. Apart from scaffold-based approaches, which are also employed under normal gravity conditions on Earth, microgravity offers unique conditions to facilitate a scaffold-free development of three-dimensional multicellular aggregates or spheroids and even organotypic tissue. So far, the underlying mechanisms of the observed spontaneous cell aggregation are not yet known, but they are subject to intensive investigation in the gravitational biology community. This knowledge can contribute to an optimization of three-dimensional tissue growth on different microgravity platforms and to the understanding of scaffold-free tissue engineering. Additionally, these constructs provide an efficient tool for downstream experiments such as drug testing and could be used as a replacement for *in vivo* models, thereby reducing the need for animal testing. Furthermore, future applications such as medical transplants are possible. This chapter will present an overview of the current state of microgravity-based tissue engineering.

Keywords Microgravity • Tissue Engineering • Spheroids • Cartilage • Bone • Endothelium

6.1 Introduction

Tissue engineering, a term first coined at the National Science Foundation Forum on Issues, Expectations, and Prospects for Emerging Technology Initiation, held at Granlibakken Resort, Lake Tahoe, California, in February 1988 and later refined by Robert Langer and Joseph P. Vacanti, is defined as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of

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biological substitutes that restore, maintain, or improve tissue function" employing the use of isolated cells or cell substitutes, tissue-inducing substances or cells placed on or within matrices (Langer and Vacanti 1993).

Early experiments under real microgravity (r-µ*g*) in space on board different Space Shuttle missions revealed that weightlessness has an influence on the aggregation behavior of human cells. Tschopp et al. found that suspended human embryonic kidney cells tended to attach to carrier microbeads (Tschopp et al. 1984), while Dintenfass observed an aggregation of red blood cells in space (Dintenfass 1986). These results indicated that microgravity might be beneficial for the formation of three-dimensional cell aggregates and led to further studies, investigating this phenomenon more thoroughly.

However, the increasing interest in the application of microgravity and the low availability of actual space flight opportunities meant that studying µ*g* on Earth soon also came into focus. Unfortunately, because of the very short µ*g* exposure time during parabolic flights (22 s) or sounding rocket missions (6 min), these two options are only of limited use for tissue engineering purposes. Therefore, devices for the simulation of microgravity (s-µ*g*) have also been employed since the very early stages of µ*g*-assisted tissue engineering. Most prominently, the NASA-developed Rotating Wall Vessel (RWV) bioreactor (Klaus 2001; Schwarz et al. 1992; Hammond and Hammond 2001) has been used for cells (with or without scaffolds) in suspension, while the Random Positioning Machine (RPM) (Borst and van Loon 2008; van Loon 2007) or the fast-rotating clinostat (FRC) (Eiermann et al. 2013) were preferred for adherent cell cultures. All these machines keep the samples in constant motion/rotation. The RWV counteracts the gravitational vector by rotating the circular culture vessel around a horizontal axis at a speed where the upward fluid flow of the medium and the downward sedimentation of the cells are a balance. This keeps the cells in a state of constant free fall. The FRC and the RPM rotate the culture flasks around one or all three axes in space leading to a mean annulled influence of the gravitational vector over time.

Under normal gravity conditions, isolated cells cultured in regular culture flasks will only grow in a monolayer (2D). In order to produce three-dimensional tissue constructs, it is therefore often necessary to introduce a so-called "scaffold", a structure that provides a surface for the cells to attach to, determines the shape and contributes to the overall mechanical stability of the generated tissue. Scaffolds are usually made from materials such as hydroxyapatite (HA), D,L-polylactic-polyglycolic acid (PLGA), bioactive glass, L-polylactic acid (L-PLA), polycaprolactone (PCL) or poly(ethylene glycol)-terephthalate (PEG/PBT) (Hollister 2005; Dutta et al. 2017). However, while helping the cells to assemble in a 3D structure in the initial phase of the tissue engineering process, the scaffolds might eventually pose some problems in the long run, such as unforeseen immunologic problems, a distorted structure of the newly formed tissue or an altered mechanical resilience compared to natural tissues. Therefore, the ultimate aim of tissue engineering is the *de novo* formation of scaffold-free, functional, organotypic tissue constructs. Employing microgravity-based tissue engineering techniques might be a step further in this direction (Grimm et al. 2014).

6.2 Tissue Engineering in Simulated Microgravity

A wide spectrum of different cell and tissue types has been used for tissue engineering studies using s-μ*g* on devices such as the RWV or the RPM. Compared to experiments in space, they have the advantages of a higher number of replicates, better control of the environment (temperature, humidity, atmospheric CO_2 concentration), a higher throughput of samples, easier accessibility of suitable facilities and highly reduced costs. On the other hand, it has to be considered that both machines can only approximate r-μ*g* conditions to a certain extent, as residual acceleration, shear forces and disturbances by bubbles are inherent to their functional principle (Wuest et al. 2015; Hammond and Hammond 2001; Lappa 2003). Nevertheless, s-μ*g*-based techniques have been the methods of choice for the majority of tissue engineering approaches.

6.2.1 Cartilage

In 1991, the first report of cartilage tissue engineering in s-μ*g* showed that rat embryonic limb mesenchymal cells growing on microcarrier beads in a RWV bioreactor eventually differentiated into functional chondrocytes, producing Alcian-blue positive matrix. Furthermore, over the 65-day experiment duration cells and microcarriers aggregated and the newly formed 3D structures kept increasing in size (Daane et al. 1991; Duke et al. 1993).

Similar observations were made in several following experiments, where in a RWV, chondrocytes of different origins seeded on polymer scaffolds formed macroscopically large (with lengths of each edge in the range of several mm) three-dimensional aggregates. The resulting tissues were very similar to natural cartilage, exhibiting comparable cell densities, glycosaminoglycan (GAG) and collagen II percentages. Furthermore, tissue constructs deriving from s-μ*g* conditions were mechanically and structurally superior to those generated in spinner flasks or in Petri dishes (Baker and Goodwin 1997; Freed et al. 1998; Freed and Vunjak-Novakovic 1997; Falsafi and Koch 2000; Gorti et al. 2003; Wu et al. 2013). It could also be shown that TGF-β_1 supplementation of the growth medium (5 ng/mL) resulted in an improved proteoglycan production of rat articular chondrocytes cultured on three-dimensional macroporous PLGA sponges for 4 weeks in a RWV (Emin et al. 2008).

The first scaffold-free generation of cartilage tissue in s-μ*g* was reported by Conza et al. (2001). As a preparation for a space flight experiment, chondrocytes were seeded into a specially designed hardware intended for use on the ISS and were cultured on an RPM for up to 3 weeks. The culture chamber geometry was cylindrical with a diameter of 8 mm and a height of either 8 or 2 mm. Cartilage tissue constructs obtained from the RPM were round in shape, in contrast to those from static controls, whose shape followed that of the culture chamber. The chondrocytes also exhibited a more ordered arrangement than those grown in 1*g*. Later results, however, showed that cartilage grown in the same hardware on the ISS was inferior to the material from the ground controls and that the RPM samples had an intermediate quality (Stamenkovic et al. 2010).

Scaffold-free engineering of cartilage tissue has also been demonstrated using dedifferentiated chondrocytes in an RWV bioreactor. After 90 days of culture, a dense collagen-II- and proteoglycan-rich cartilaginous tissue was found consisting of highly metabolically active chondrocytes (Marlovits et al. 2003).

Another scaffold-free approach was used by Aleshcheva et al. (Aleshcheva et al. 2016, Grimm et al. 2014). Adherent chondrocytes were cultured for up to 21 days on an RPM. At the end of this period, some chondrocytes had spontaneously detached from the bottom of the culture flasks and formed multicellular spheroids suspended in the tissue culture medium. Their size was also in the mm range, but overall smaller in comparison to their scaffold-supported counterparts. First studies to elucidate the possible mechanisms of this scaffold-free cartilage growth employing parabolic flights and further experiments on the RPM indicated that genes involved in the mechanical properties of the cells as well as adhesion, growth and apoptosis were regulated upon exposure to μ*g*. Furthermore, it could be shown that during cultivation on the RPM the chondrocytes switched from collagen I and –X production towards collagen II, chondroitin sulphate and aggrecan production (Ulbrich et al. 2010; Aleshcheva et al. 2013, 2016).

Besides employing already differentiated chondrocytes, it was also demonstrated by several groups that mesenchymal stem cells (MSCs) could be induced to differentiate into a chondrocyte phenotype in RWV bioreactors. A scaffold-free method has been described by Ohyabu et al. (2006), generating large ($1.25 \pm 0.06 \times 0.60 \pm 0.08$ cm) cartilaginous tissue constructs from suspended rabbit bone marrow cells cultivated in an RWV for 3 weeks. Collagen I, II, safranin-O and toluidine blue staining together with the gene expression patterns of aggrecan, and collagens I and II as well as the glycosaminoglycan/DNA ratio confirmed the cartilaginous properties of the tissue. The possible role of TGF-β_1 is still debated, as one study showed no influence of this molecule on the s-μ*g*-induced differentiation of MSCs into chondrocytes (Luo et al. 2011), while other authors showed that s-μ*g* and TGF-β_1 synergistically promote the differentiation into chondrocytes by activating the p38 MAPK pathway (Yu et al. 2011). However, very recently, it was reported that mesenchymal stem cells differentiated into chondrocytes without the use of an exogenous growth factor when cultivated on decellularized cartilage ECM-derived particles in a RWV for 21 days. The resulting cartilage microtissue aggregates. Most interestingly, these constructs, when implanted with fibrin glue into a rat model for cartilage defects, were shown to improve and accelerate joint function recovery and cartilage repair in comparison to the microtissue constructs or fibrin glue alone.

6.2.2 Thyroid Cancer Spheroids

S-μ*g* has been identified as a means to produce spheroids from different types of malignant cells early on. Multicellular tumor spheroids (MCTSs) offer many possibilities for further studies of tumor development, metastasis, host-tumor interactions and drug testing, among others (Jessup et al. 1993; Ingram et al. 1997).

Currently, the majority of spheroids used for these kinds of analyses are still generated under classical 1*g*conditions, as illustrated by a selection of the most recent publications (Halfter et al. 2016; Akasov et al. 2016; Ravi et al. 2016; Wang et al. 2016). More in-depth reviews are given in Mehta et al. (2012) and Wang et al. (2014). However, s-µg-generated spheroids might be superior to their 1g counterparts, as culture conditions allow for a more physiological structure of the tissue constructs, undisturbed by any potentially interfering sedimentation force, thereby simplifying the translation from *in vitro* results to *in vivo* applications. Due to the diversity of different MCTSs generated under s-µ*g*, this paragraph will focus on thyroid cancer cells.

Using the RPM, Grimm et al. were successful in generating MCTSs from the adherent thyroid carcinoma cell lines ML-1 and FTC-133 (Grimm et al. 2002; Pietsch et al. 2011). It was found that s-µ*g* induced increased apoptosis in both cell lines, possibly reflecting the reduction of thyroid function observed in astronauts (Strollo 1999). Both proteomic and genomic analyses of FTC-133 MCTSs vs. 1g control cultures revealed that during spheroid formation the cells express fibronectin-binding surface proteins, thereby strengthening the cell-to cell adhesion (Pietsch et al. 2011), and that the genes *IL-6*, *IL-8*, *OPN*, *TLN1*, *CTGF*, *NGAL*, *VEGFA*, *IL17*, *VEGFD*, *MSN*, *MMP3*, *ACTB*, *ACTA2*, *KRT8*, *TUBB*, *EZR*, *RDX*, *MSN*, *PRKCA*, *MMP9*, *PAI1* and *MCP1* were generally regulated in such a manner that they upregulated genes coding for proteins, which promote 3D growth (angiogenesis) and prevent excessive accumulation of extracellular proteins, while gene coding for structural proteins is downregulated in MCTSs (Pietsch et al. 2011; Grosse et al. 2012; Warnke et al. 2014; Kopp et al. 2015; Riwaldt et al. 2015a, 2016).

6.2.3 Bone

Bone tissue is one of the most researched aspects in the field of tissue engineering in µ*g*. So far, however, all efforts have been confined to experiments in s-µ*g*.

The first step in bone tissue engineering was reported in 1998 by Qiu et al. (1998). Secondary rat marrow stromal cells were cultured for 2 weeks on Cytodex-3 microcarrier beads in an RWV and formed spherical aggregates exhibiting mineralization as well as alkaline phosphatase activity and collagen type I and osteopontin expression. Over the years, the technique for bone tissue engineering was further refined, but in principle, it is always a variation of using either osteoblasts or mesenchymal stem cells grown on different scaffold (interconnected porous HA, porous PLGA, bioactive glass-polymer composites, human bioderived bone scaffolds, alginate or gelatin) cultures in an s-µ*g* device, usually an RWV. Most studies showed that the s-µ*g*-derived tissue was comparable to natural bone and usually superior to engineered tissue from static cultures, as evidenced by their greater *in vivo* effectiveness in repairing bone lesions in animal models (Sikavitsas et al. 2002; Nishikawa et al. 2005; Song et al. 2006, 2007, 2008; Hwang et al. 2009; Lv et al. 2009; Jin et al. 2010; Cerwinka et al. 2012; Ulbrich et al. 2014).

6.2.4 *Endothelium*

Endothelial cells, the inner lining of the blood vessels, play an important role in many physiological processes in the human body, most notably in the regulation of blood pressure. Lesions in the endothelium can lead to life-threatening complications, such as infarctions. Therefore, endothelial repair/blood vessel replacement is an important topic in modern medicine. Furthermore, for complicated (micro) surgical procedures it could be advantageous to produce autologous vessels to circumvent possible rejections of important grafts.

Three-dimensional endothelial cell constructs in a RWV were first generated by Sanford et al. (2002). Bovine aortic endothelial cells were first seeded onto Cytodex-3 microcarrier beads and then cultivated in s-µ*g* for 30 days. The authors found large tissue-like aggregates consisting of at least 20 beads and viable cells of typical endothelial cell morphology, forming multilayered sheet-like structures separated by a zone of matrix material. The cells showed tenfold enhanced NO production compared to Spinner flask control cultures, which was inducible by l-arginine and blockable by L-NAME, indicating a physiological behavior. Furthermore, they showed increased barrier properties.

In 2005, CD34+ human umbilical cord stem cells were cultured in s-µ*g* using RWVs with or without Cytodex-3 microcarrier beads for 14 days. The growth medium contained 50 ng/mL vascular endothelial growth factor (VEGF). Interestingly, on day 4 the cells cultured in the absence of microcarrier beads formed three-dimensional aggregates resembling tubular structures, whereas in the bead-containing RWVs only amorphic cell clusters were found. FACS analyses revealed that the cells in the tubular structures expressed endothelial markers such as CD34, CD31 and flk1 and microscopically they exhibited the morphologies of vascular endothelial-like cells and spindle cells (Chiu et al. 2005). In accordance with this study, it was later confirmed that s-µ*g* conditions in a clinostat lead to differentiation of mesenchymal stem cells into an endothelial phenotype, expressing typical endothelial markers such as Flk-1 and vWF (Zhang et al. 2013).

Using the RPM to culture the immortalized endothelial cell line EA.hy926, a fusion of human umbilical vein endothelial cells (HUVECs) with a thioguanine-resistant clone of A549 adenocarcinoma cells (Edgell et al. 1990), with and without a supplementation of 10 ng/mL VEGF in the growth medium for 72 h, Infanger et al. (2006) observed an initial increase in the expression of extracellular matrix proteins induced by both s-µg and VEGF alone, which was further augmented by s-µ*g* after 12 h. In addition, s-µ*g* induced apoptosis beginning from four h culture time and increasing until 72 h, while VEGF reduced the apoptosis rate. After 72 h, the authors also found that many non-apoptotic cells had formed tube-like aggregates. These tubes were further characterized and it was found that adherent Ea. hy926 cultured on an RPM began to form small colonies by spreading over neighboring cells. From these colonies, tube-like structures emerged after 2 weeks of

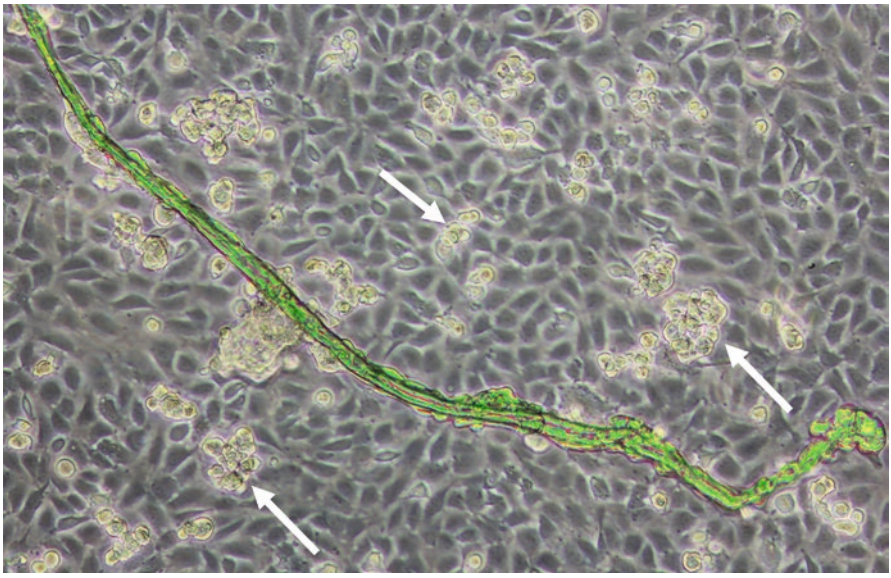

Fig. 6.1 Endothelial tubular structure after 21 days culturing on the RPM. All three types of cell growth observed on the RPM are shown. In the background a 2D monolayer of adherently, growing endothelial cells can be seen, while two kinds of 3D aggregates are also present. *White arrows* point out multicellular spheroids, a large tubular structure similar to an intima, is highlighted in *green* for better visibility

cultivation, which formed a defined lumen and continued to elongate over the course of 2 more weeks of RPM culture. The tube walls resembled vascular intimas and consisted of a single layer of cells (Fig. 6.1), which produced more β1-integrin, laminin, fibronectin and α-tubulin than 1*g* control cells.

It can therefore be assumed that the specific s-μ*g* culture conditions on an RPM offer a unique opportunity to study the mechanisms of 3D vessel development (Grimm et al. 2009). The first studies to elucidate the mechanism of tube formation hinted at an involvement of phosphokinase cα and of an interaction network formed by the genes *RDX*, *EZR*, *MSN*, *GSN*, *CALD1*, *SPTAN1*, *VIM*, *TLN1*, *ITGB1*, *CAV1*, *ANXA2*, *ICAM1*, *ENG*, *SERPINE1*, *IL6* and *IL8* (Grimm et al. 2010; Ma et al. 2013).

6.3 Tissue Engineering in Real Microgravity

Compared to tissue engineering approaches on Earth in an s-μ*g* environment, conducting such projects in space is a far more technically, logistically and, of course financially challenging endeavor. Therefore, their absolute number is relatively small.

6.3.1 Cartilage

The first attempts to grow cartilage tissue constructs during space missions were undertaken by Freed et al. (Freed et al. 1997; Saltzman 1997). This was a long-term experiment lasting a total of 7 months. The authors first generated three-dimensional cell-polymer constructs from bovine articular chondrocytes and polyglycolic acid scaffolds in rotating bioreactors on Earth over a period of 3 months. Afterwards, one reactor containing ten 3D constructs was transported to the *MIR* space station and the cultivation continued under r-µ*g* for a further 4 months. In parallel, a second bioreactor with ten constructs was left on Earth in 1*g* and was operated for the same time. Under both gravitational conditions, functional and viable cartilaginous constructs emerged. However, the r-µ*g* samples tended to possess an overall rounder shape, smaller size and reduced mechanical stability when compared to those grown on Earth (Freed and Vunjak-Novakovic 1997; Freed et al. 1999).

A scaffold-free approach was used for the generation of neocartilage derived from porcine chondrocytes. The cells were seeded in cylindrical culture chambers and subsequently exposed to r-µ*g* on board the ISS, s-µ*g* on an RPM and, as a control, 1g in a stationary set-up on Earth (Stamenkovic et al. 2010; Conza et al. 2001). The experiment lasted for 16 days, after which the tissue was subjected to histological and gene expression analyses. The authors found that, compared to those from s-µ*g* and 1*g*, the samples from the ISS showed a weaker stain for extracellular matrix. The ISS samples also possessed a higher collagen II/I expression ratio than control tissue. On the other hand, aggrecan/versican expression and cell density were increased in 1*g* tissues compared to both r- and s-µ*g*. These results are in accordance with those found by Freed et al. (1997) and seem to reflect the observed average loss of about 8% of cartilage thickness after 14 days of mechanical unloading during a 6-degree head-down-tilt bedrest in young healthy subjects (Liphardt et al. 2009).

6.3.2 Thyroid Cancer Spheroids

Due to its tolerance to culture temperatures well below the ideal 37 °C, the human follicular thyroid cancer cell line FTC-133 was chosen for two space flight missions, aimed at generating MCTSs under r-µ*g*. The first mission, SIMBOX on Shenzhou-8 in 2011, was conducted for 10 days, using a specially designed cell culture hardware by Airbus Defence and Space. After the flight, several MCTSs were found inside the culture vessels, which were considerably bigger (about 4–5 mm in diameter) than comparable MCTSs generated on an RPM in a parallel control experiment (Pietsch et al. 2013). Subsequent analyses of the MCTSs and the cell culture supernatants suggested that *EGF* and *CTGF* might be involved in r-µ*g*-induced MCTS formation and that a regulation of *IL6*, *IL8*, *IL15*, *OPN*, *VEGFA*, *VEGFD*, *FGF17*, *MMP2*, *MMP3*, *TIMP1*, *PRKAA* and *PRKACA* in r-µ*g* (and s-µ*g*

RPM control experiments) might shift the cells towards a less aggressive phenotype (Pietsch et al. 2013; Ma et al. 2014).

The second space-flown experiment was CellBox-1 in 2014, essentially designed as a replicate of the SIMBOX experiment, this time conducted for 10 days in the ESA Columbus module of the ISS. However, due to launch delays, the protocol for cell culture had to be adapted to the new situation. This led to an overgrowth of cells on the ground, ultimately preventing the formation of MCTSs in space. However, this led to the finding that an increased production of extracellular matrix-related proteins has the potential to prevent spheroid formation in r-µ*g* (Riwaldt et al. 2015b).

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Chapter 7
Cancer Research in Space

Marcus Krüger, Johann Bauer and Daniela Grimm

Abstract Real and simulated microgravity (μ*g*) created either by spaceflights or by special Earth-based devices provide a unique environment for studying and influencing tumor cell processes. By investigating growing cancer cells in μ*g*, researchers have shown that μ*g*-conditions change the microtubules and mitochondria of cancer cells, modify the production and structure of cytoskeletal and extracellular matrix proteins, induce apoptosis and change the secretome. It was also observed that some of the cells form three-dimensional structures, which resemble either spheres or the organ structures from which the cells originate. Cancer cells included in "spheroids" look different and behave differently to those grown on a flat surface, more closely mimicking tumor biology in human organisms. Results may be used to rethink cancer research on Earth with the aim of developing new drugs and cancer treatment strategies. The following chapter summarizes research on cancer cells under the influence of real and simulated μ*g* with a special focus on thyroid cancer, breast cancer and malignant melanoma.

Keywords Microgravity • Multicellular Spheroids • Apoptosis • Cytoskeleton • Extracellular Matrix • Angiogenesis

7.1 Introduction

Cancer is a group of diseases that show unrestrained division of cells, which in turn is caused by "misregulated" genes. In contrast to benign tumors, a malignant tumor means the cancer cells can spread through the blood and/or lymph systems. These cancer cells form new tumors (metastatic tumors) in other parts of the body. Up to now, it has not been fully understood how and why an uncontrolled proliferation and spreading with distant metastasis takes place; and this lack of understanding often stands in the way of an effective cure. One intriguing finding of space research is that gravity, or the lack thereof, has a significant effect on the regulation of cell growth (Pietsch et al. 2011a).

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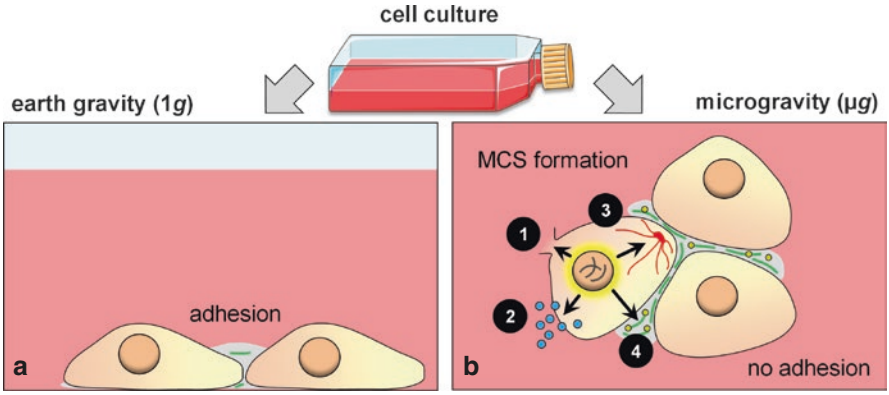

Fig. 7.1 Effects of microgravity on cell cultures. (**a**) "Normal" cell culture under 1*g*: cells grow attached to the ground of the culture flasks. (**b**) Cell culture under µ*g*: some of the cells grow attached to each other. The alteration of gravity influences gene expression (highlighted in *yellow*) and leads to (1) enhanced apoptosis, (2) changes in cell signaling, (3) rearrangement of the cytoskeleton, and (4) alteration of the extracellular matrix. *Parts of the figure were drawn by using pictures from Servier Medical Art*

In particular, microgravity has been shown to have far-ranging effects on cell functions (modifications of the transcriptome, proteome and secretome) (Grimm et al. 2011; see also Fig. 7.1), including:

- "Programmed" cell death (apoptosis)
- Secretion of cytokines and growth factors
- Arrangement of the cytoskeleton
- Composition and structure of the extracellular matrix (ECM)

In the microgravity environment of space, tumor cells detach from the bottom of plastic dishes and assemble—like other cells—into multicellular three-dimensional constructs (Ingram et al. 1997). These "multicellular spheroids" (MCSs) mimic small metastases and represent the *in vivo* structure of tumors more closely than monolayer cell cultures can do (Grosse et al. 2012; Grimm et al. 2014; Hirschhaeuser et al. 2010; Kunz-Schughart 1999). They are used for studies on biological processes and for pharmacological testing (Grimm et al. 1997, 2014). MCSs are not as complex as natural tumors but are able to resemble parts of the organ from which the cells have been derived (Kopp et al. 2016).

7.2 Contribution of Space Research to Cancer Research

In 1998, during the STS-90 mission, primary cultures of human renal cortical cells were cultured for 6 days aboard the space shuttle *Columbia* before they returned to Earth for analysis. Hammond et al. showed that 1632 of the 10,000 analyzed genes changed their activation status relative to ground controls (Hammond et al. 2000). This was the first experiment to show that reduced gravity can affect a wide range

of genes. These findings led to speculations that weightlessness could also trigger cancer cells to change the expression of numerous proteins, which could be the basis for the development of new targets for drugs (Frandsen et al. 2016).

As spaceflights are rare and extremely expensive, different ground-based facilities have been developed aimed at achieving functional weightlessness (simulated microgravity; s-μ*g*) for analyzing the molecular and biochemical mechanisms affected by microgravity (Becker and Souza 2013): Fast-rotating Clinostats (FRCs) (Eiermann et al. 2013), Rotating Wall Vessels (RWVs) (Klaus 2001) and Random Positioning Machines (RPMs) (Borst and van Loon 2008; van Loon 2007) allow a cost-efficient preparation of spaceflights as well as intensive research in stand-alone studies (Fig. 7.2d). They have been shown to imitate microgravity effects for several, but not all, experimental conditions (Herranz et al. 2013).

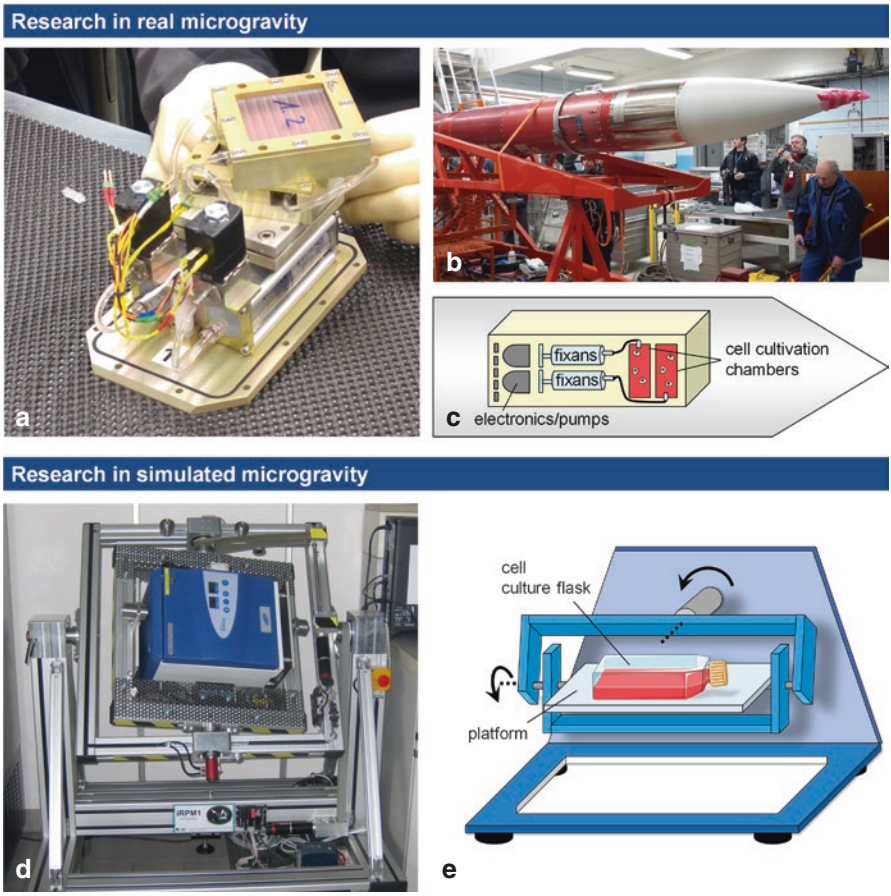

Fig. 7.2 Research on cancer cells under the effects of microgravity. (**a**) Flight hardware with cell cultivation chambers for experiments in unmanned missions. (**b**) TEXUS sounding rocket. (**c**) Schematics of the flight hardware for cancer cell cultivation on a TEXUS rocket mission. (**d**) Random Positioning Machine (RPM). (**e**) Schematics of a RPM parts of the figure were drawn by using pictures from Servier Medical Art

The DLR parabolic flight campaigns (PFCs) and rocket missions like the European/German sounding rocket program TEXUS allow periodical studies under real microgravity (r-µ*g*) for periods of seconds to minutes (Fig. 7.2).

Table 7.1 summarizes previous investigations of different cancer types under the effects of microgravity. Research in cooperation with the DLR is given a closer look in the following chapters.

Table 7.1 Studies on cells of different human cancer types

Cancer type	Most important findings	s-µ*g*	r-µ*g*
T-cell leukaemia	Altered cytoskeletal gene expression and increased apoptosis (Lewis et al. 1998, 2001); Fas/APO-1 protein increased (Cubano and Lewis 2000)		x
Bone cancer (osteosarcoma)	Activation of Egr-1 and NF-κB (Granet et al. 2001); repression of TNFα-dependent activation of NF-κB (Kobayashi et al. 2000); vector-averaged gravity could cause the death of osteoblasts during the first 24 h of clinorotation (Sarkar et al. 1999)	x	
Brain cancer (malignant glioma)	Inhibition of growth and mitochondrial activity; deceleration of mitosis; enhanced chemosensitivity to cisplatin (Takeda et al. 2009)	x	
Cervical cancer	MCS formation; co-culture forms tubular structures (Chopra et al. 1997)	x	
Colon cancer	MCS formation; co-culture with fibroblasts (Goodwin et al. 1992); 3D growth *in vitro* simulates tumor function *in vivo* (Jessup et al. 1997); reduced apoptosis and increased differentiation (Jessup et al. 2000)	x	
Liver cancer (hepatocellular carcinoma)	MCS formation; AD: actin stress fibres, upregulation of structural genes; MCS cortical actin, regulation of metabolic and synthetic genes (Chang and Hughes-Fulford 2009); MCSs model many clinical pathological features of hepatocellular carcinoma *in vivo*, including cancer cell morphology, tissue ultrastructure, protein production and secretion, glucose metabolism, tissue-specific gene expression and apoptosis (Tang et al. 2011)	x	
Lung cancer	Increase in apoptosis, stem cells lose their stemness features: decrease in ALDH, downregulation of NANOG and OCT4 (Pisanu et al. 2014); increased migratory ability of two cell lines (Chung et al. 2016)	x	
Ovarian cancer	MCS formation (Becker et al. 1993); 3D culture of a mixed Mullerian tumor mimics the *in vivo* morphology (Goodwin et al. 1997)	x	
Prostate cancer	Less aggressive cells with higher percentage of G1-phase cells, larger bead aggregates, enhanced development of filopodia and microvilli-like structures on the MCS surface; stronger staining for select cytoskeletal proteins (cytokeratins 8/18, actin, vimentin) (Clejan et al. 1996); induced changes in lipid second messengers and signal transduction (Clejan et al. 2001); co-culture with prostate fibroblasts (Zhau et al. 1997); co-culture of human prostate cancer and bone cells (Wang et al. 2005); co-cultured prostate and bone stromal cells accelerate cancer growth and metastasis (Sung et al. 2008)	x	

Table 7.1 (continued)

Cancer type	Most important findings	s-μ*g*	r-μ*g*
Neuroblastoma	*In vitro* aggregation kinetics and organoid morphology correlate with MYCN expression (Redden and Doolin 2011); flavonoids protect against induced oxidative stress (Qu et al. 2010)	x	
Astrocytoma	Reduction of adenylyl cyclase type 6 expression (Matsuoka et al. 2007)	x	
Epidermoid carcinoma	Inhibition of early EGF-induced signal transduction (Rijken et al. 1991, 1992b); alteration of the cytoskeleton (Rijken et al. 1992a)	x	
Leydig tumor	Increased progesterone production (Kaneko et al. 2008)	x	
Testicular cancer (seminoma)	Cytoskeleton modification; induction of autophagy (Ferranti et al. 2014)	x	
Thyroid cancer	See Sect. 7.3	x	x
Breast cancer	See Sect. 7.4	x	x
Skin cancer	See Sect. 7.5	x	

Cells were exposed either to simulated microgravity in ground-based facility devices (RPMs, clinostats; s-μ*g*) or to real microgravity (PFCs, sounding rockets, space; r-μ*g*)

7.3 Studies on Thyroid Cancer

Thyroid cancer is the most common type of endocrine neoplasia (see Fig. 7.3). The incidence in 2012 was estimated to be 298,102 new cases worldwide, representing 2.1% of all cancers (Torre et al. 2015). The diagnosis of thyroid cancer has risen rapidly over the past three decades (Carling and Udelsman 2014), due to an improved detection rate of, in particular, small tumors and a substantial increase in incidence. The leading causes are thought to be the population's increased exposure to radiation and other currently unknown carcinogens (Lorusso and Newbold 2015). Ninety-four percent of all thyroid cancer types are well-differentiated thyroid carcinomas (DTCs) and respond well to treatment. Approximately 20% of the patients experience local recurrence and 10% distant metastasis. A recurrent DTC often becomes less differentiated (then it is called "poorly differentiated thyroid cancer" or PDTC). PDTC loses its iodine uptake capability and therefore consequently the option for radioactive iodine treatment. As PDTC is highly unlikely to be curable (Laursen et al. 2016), the 10-year survival rate drops rapidly. As chemotherapeutic drugs have a significant toxicity and show only transient and limited response rates (Baudin and Schlumberger 2007), the development of new treatment strategies and the search for new target proteins are important topics.

Grimm and co-workers were the first to focus on thyroid cancer cells under the effects of simulated microgravity (Grimm et al. 2002a, b), with the aim of broadening our knowledge about their migration and aggregation behavior. In their early studies, they used the ML-1 cell line, originating from a dedifferentiated follicular thyroid carcinoma relapse of a 50-year-old female patient (Schonberger et al. 2000).

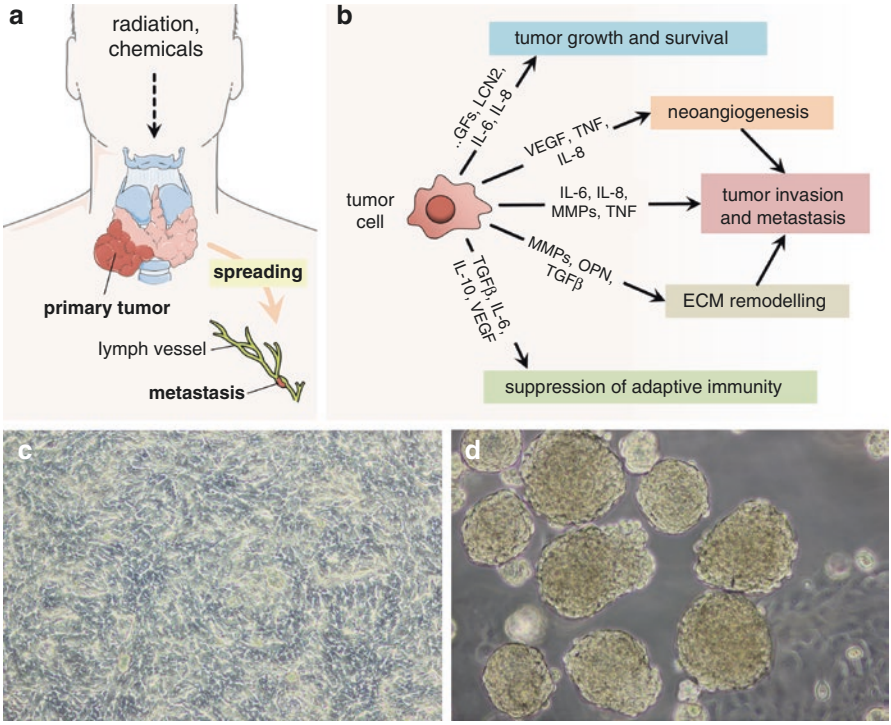

Fig. 7.3 Thyroid cancer and thyroid cancer cells. (**a**) Schematics of a thyroid carcinoma. (**b**) Factors and processes in tumor spreading. (**c**) Adherent cells of a poorly differentiated thyroid carcinoma. (**d**) MCS of the same thyroid carcinoma cells exposed to s-μ*g* for 7 days on an RPM. *Parts of the figure were drawn by using pictures from Servier Medical Art*

During exposure to the RPM thyroid cancer cells detached from the surface of the culture flasks and formed multicellular spheroids. Grimm et al. detected an increase of extracellular matrix proteins and TGF-β1 (Grimm et al. 2002b) and found hints of early programmed cell death, such as chromatin condensation, membrane blebbing, loss of nuclear envelope and cellular fragmentation into apoptotic bodies. In this stage, the amount of cytoskeletal intermediate filament protein vimentin was increased (Grimm et al. 2002a) and this finding was consistent with previously discovered abnormalities in actin stress fibers and microtubuli in Jurkat cells flown on the Space Shuttle (Schatten et al. 2001). Further investigations showed elevated amounts of the apoptosis-associated Fas protein p53 and Bax. Caspase-3 was clearly upregulated. This was a clear hint that simulated microgravity induces early programmed cell death using different pathways of apoptosis (Kossmehl et al. 2002). By using a mitochondria-rich carcinoma cell line Kossmehl et al. could show that gravitational unloading affects the mitochondria and thereby may trigger apoptosis (Kossmehl et al. 2003).

In a first proteome analysis Pietsch et al. compared ML-1 cells cultured under 1*g* with those on the RPM. When cells had been cultured under s-μ*g* many proteins

showed different Mascot scores and proteins related to cell growth (glutathione S-transferase P, nucleoside diphosphate kinase A, heat shock cognate 71 kDa protein) were enhanced (Pietsch et al. 2011b).

7.3.1 The Cytoskeleton May Act as a "Gravisensor"

When ML-1 cells were investigated during parabolic flight maneuvers a gene array analysis revealed 2430 significantly changed transcripts after 22 s in real microgravity. In addition, the cytoskeleton compounds F-actin and cytokeratin were altered (Ulbrich et al. 2011). Furthermore, *ACTB* and *KRT80* mRNAs were significantly upregulated.

Investigations on a Vibraplex device and a Short-Arm Human Centrifuge (SAHC) in Cologne demonstrated that neither hypergravity nor vibrations could induce the expressional change of *ACTB* and *KRT80* (Ulbrich et al. 2011). So, microgravity seems to alter gene expression patterns and the cytoskeleton of ML-1 cells very early after exposure to µ*g*. A similar finding was made by Infanger et al. when they observed papillary thyroid carcinoma cells exposed to s-µ*g*. After 30 min of clinorotation, vimentin and cytokeratin were highly disorganized, and microtubules (α-tubulin) did not display their typical radial array. After 48 h, the cytoskeletal changes were nearly reversed (Infanger et al. 2006). This finding argues for the suggestion of Vorselen et al. that the cytoskeleton may act as a "gravisensor" in cells (Vorselen et al. 2014). In life-cell imaging experiments with a FLUMIAS fluorescence microscope during the 24th DLR PFC and the TEXUS-52 rocket mission, cytoskeletal changes of FTC-133 cells could be visualized. These changes occur rapidly after entrance into microgravity, which confirms the results obtained in earlier studies. Under the microscope, disturbance of F-actin bundles was detected as well as the formation of filopodia- and lamellipodia-like structures (Corydon et al. 2016).

7.3.2 The FTC-133 Cell Line Is More Suitable for Space Experiments

For experiments on space missions, cells must be more unsusceptible to rough cultivation conditions (e.g. incubation at 20 °C). Preliminary tests made FTC-133 cells the first choice. The FTC-133 cell line is the first stable cell line of a human follicular thyroid carcinoma and was derived from a 42-year-old male suffering from a poorly differentiated thyroid carcinoma (PDTC) (Goretzki et al. 1990). This type of thyroid cancer has a very unfavorable prognosis (Durante et al. 2006). FTC-133 cells form large 3D aggregates when they are cultured under real and simulated microgravity (Grosse et al. 2012; Pietsch et al. 2011c, 2013a; Warnke et al. 2016). Grosse et al. showed in 2012 that several cytokines, such as IL-6, IL-8 and

osteopontin (OPN), are involved together with NF-κB subunit p65 (RelA) in the three-dimensional growth of follicular thyroid cancer cells exposed to an RPM (Grosse et al. 2012). Compared with normal thyroid cells, thyroid cancer cells seem to react to s-μ*g* much earlier with a production of OPN, which could explain the larger spheroids of cancer cells (Kopp et al. 2015).

The proteome analysis of FTC-133 thyroid cancer cells revealed a huge number of housekeeping proteins (Pietsch et al. 2013b). In addition, it suggested that the expression of integrin-α5 chains together with integrin-β1 chains and proteins such as myosin-10 and filamin-B binding fibronectin is what causes FTC-133 to form larger and more numerous MCSs under s-μ*g* than other comparable thyroid cancer cells (Pietsch et al. 2011c).

7.3.3 *The First Space Flight of Thyroid Cancer Cells:* r-μ*g* vs. s-μ*g*

As a part of the Sino-German Shenzhou-8/SIMBOX mission experiment in 2011, FTC-133 cells were exposed to r-μ*g* in space for 10 days. In an automated culturing system cells were able to form extraordinarily large MCSs. *EGF* and *CTGF* genes were upregulated in space similarly to on the RPM, but both genes were expressed much more highly in space (Pietsch et al. 2013a). Microarray analyses revealed 2881 significantly regulated transcripts in cells cultured on the rocket (Ma et al. 2014). These genes code for products involved in several biological processes, characterized by the gene ontology terms apoptosis, cytoskeleton, adhesion/extracellular matrix, proliferation, stress response, migration, angiogenesis and signal transduction. Genes and proteins involved in the regulation of cancer cell proliferation and metastasis, such as *IL6*, *IL8*, *IL15*, *SPP1*, *VEGFA*, *VEGFD*, *FGF17*, *MMP2*, *MMP3*, *TIMP1*, *PRKAA* and *PRKACA* mRNAs, were similarly regulated during space flight conditions as in cells exposed to the RPM (Ma et al. 2014). These experiments indicated that spaceflights and ground-based facilities in some aspects induce the same changes in the expression of genes and in the secretion of proteins involved in cancer cell proliferation, metastasis and survival (Fig. 7.3b). These changes shift the cells toward a less aggressive phenotype.

7.3.4 *Alteration of the Extracellular Matrix*

Cancer cells actively elaborate and remodel their extracellular matrix (ECM), which has important effects on their survival and progression (Nelson et al. 2006). Infanger and co-workers exposed carcinoma cells (ONCO-DG 1) to simulated microgravity on an RPM and demonstrated that weightlessness not only rapidly affects the cytoskeleton of ONCO-DG 1 cells but also increases the amount of ECM proteins in a time-dependent manner (Infanger et al. 2006). Notably the amount of E-cadherin

was enhanced time-dependently and the accumulation of ECM components, such as collagen types I and III, fibronectin, chondroitin sulphate, osteopontin and CD44, increased with the duration of exposure to the RPM. Secretion of lipocalin-2 (LCN2 or NGAL), which has been identified as a survival factor for thyroid neoplastic cells (Iannetti et al. 2008), was reduced in both the RPM and space flight samples of FTC-133 cells (Ma et al. 2014).

Riwaldt et al. detected an interesting group of proteins related to the extracellular matrix when they determined the proteome of FTC-133 cells, which had been flown to the International Space Station (Cellbox-1 experiment) and were cultured there for 10 days (Riwaldt et al. 2015b). In contrast to earlier experiments with FTC-133 cells exposed to microgravity, the cells obtained after the Cellbox-1 mission had not formed spheroids. The reason for this failure was that their exposure to microgravity began after the cell monolayer had reached confluence (Pietsch et al. 2013a; Riwaldt et al. 2015b). At that time, a lot of ECM was surrounding the cells (Riwaldt et al. 2015b). Following investigations brought evidence that caveolin-1 (a tumor suppressor gene candidate and a negative regulator of the Ras-p42/44 MAP kinase cascade) is involved in the inhibition of spheroid formation, when confluent monolayers are exposed to microgravity (Riwaldt et al. 2015a). In earlier studies the *CAV1* mRNA was downregulated during spheroid formation on devices simulating microgravity (Warnke et al. 2014). Bauer et al. postulated that the stability of extracellular matrix has a great influence on the formation of three-dimensional aggregates under μ*g*, as they examined genome and proteome data of different experiments in space research (Bauer et al. 2016). Lysine 6 oxidase (LOX), which supports cross-linking of collagen and elastin, was 100-fold downregulated during the Shenzhou-8 mission, when large spheroids were formed.

7.3.5 Changes in Cell Signaling

It has been suggested that cytokines play important roles in many processes associated with cancer, such as tumor cell proliferation, apoptosis, tumor invasion, angiogenesis and metastatic tumor cell dissemination (Zeng et al. 2012). Simulated microgravity influences the release of cytokines in follicular thyroid cancer cells, and the production of integrin-β_1 and talin-1, and predicts an identical effect under real microgravity conditions (Svejgaard et al. 2015). *IL6* gene activation seems to be very sensitive to physical forces in thyroid cells cultured *in vitro* as monolayers, as expression changed in μ*g* as well as in hypergravity or under vibration (Ma et al. 2013).

7.4 Studies on Breast Cancer

With 1.7 million cases in 2012, breast cancer is the second most common cancer worldwide and the most common invasive cancer in women (Torre et al. 2015). There has been a marked increase of over 20% since 2008 (Ferlay et al. 2015).

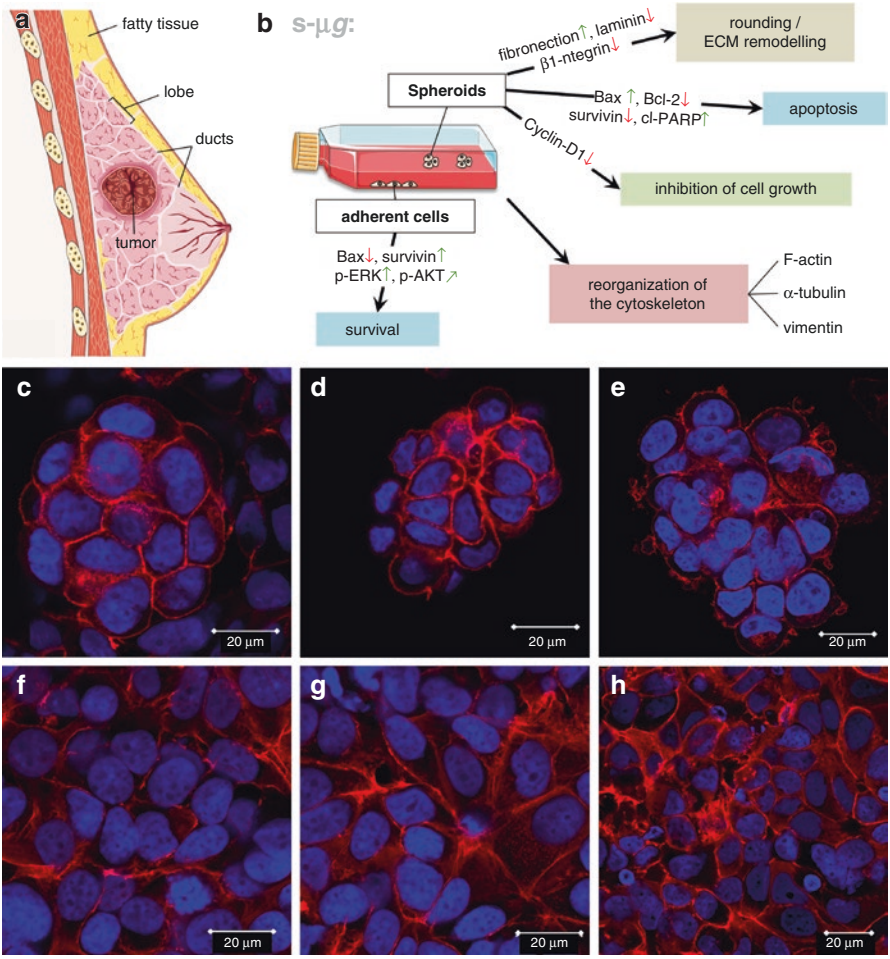

Fig. 7.4 Breast cancer and breast cancer cells. (**a**) Female breast with mammary carcinoma. (**b**) Effects on breast cancer cells when they were cultured in s-µ*g*. (**c–e**) MCS of MCF-7 cells during 5 days of clinorotation on an RPM. (**f–h**) Adherent MCF-7 cells cultured under 1*g*. Scale bar: 20 µm; *blue staining*: DAPI highlights the nucleus; *red staining*: rhodamine-phalloidin to visualize the F-actin. *Parts of the figure were drawn by using pictures from Servier Medical Art*

About 5–10% of breast cancers are thought to be hereditary. Most inherited cases of breast cancer are associated with two abnormal genes: breast cancer genes 1 and 2 (*BRCA1*, *BRCA2*) (Chen and Parmigiani 2007). Breast cancer is a highly heterogeneous disease. The most common type is ductal carcinoma, which begins in the cells of the ducts (Fig. 7.4a). Lobular carcinoma is often found in both breasts. Inflammatory breast cancer is an uncommon type that causes a warm, red and swollen breast.

The poorly invasive MCF-7 cell line was derived from a pleural effusion of a patient with metastatic mammary carcinoma. Cells are described to build up 3D

dome structures upon absolute confluence, which, however, remain attached to the bottom. Furthermore, the cells retain breast cell common features such as estrogen receptor and progesterone receptor (Soule et al. 1973).

7.4.1 Microgravity Triggers Rearrangement of the Cytoskeleton

Vassy et al. were the first scientists to investigate a human mammary carcinoma cell line exposed to microgravity. They observed changes in the cytoskeleton and the cell cycle of MCF-7 cells when these came back from a Photon capsule mission (Vassy et al. 2003, 2001). Qian et al. showed that s-μ*g* affects several cell features including cell migration and adhesion of MCF-7 cells (Qian et al. 2008). An interesting finding of this study was that the expression of vinculin, a highly conserved cytoskeletal protein that is essential for the regulation of cell morphology and migration and that can regulate the ability of cancer cells to spread to other parts of the body (Janssen et al. 2006; Nhieu and Izard 2007), was decreased after 48 h but had returned to control levels after 72 h in s-μ*g*. This indicated that simulated microgravity affects vinculin expression in a time-dependent manner (Qian et al. 2008). Li et al. demonstrated in another study that s-μ*g* impedes the cytoskeleton of MCF-7 cells by disorganizing microtubules and relocation of focal adhesion complexes and assumed that this rearrangement of the cytoskeleton might control signaling cascades and thereby govern cellular motility and migration (Li et al. 2009). Masiello et al. stated that RPM-exposed MDA MB-231 cells showed a larger rearrangement of F-actin, α-tubulin and vimentin than on-ground control cells (Masiello et al. 2014).

7.4.2 Formation of MCSs

Spheroids of human breast cancer cells were described by Becker and Blanchard, who cultured primary breast tumor cells in a RWV. Compared with fresh tumor cells the complex tissue-like spheroids exhibited significantly increased proliferative activity in conjunction with oncogene activation (expression of HER2/neu, H-ras, K-ras, p53, TGF-α, TGF-β, IL-1 and IL-6) and developed into aggressive aneuploid populations (Becker and Blanchard 2007).

Masiello et al. exposed MDA-MB-231 cells for 24 and 72 h to an RPM and reported on two populations (adherent cells and MCSs) that differed in cell processes (proliferation and apoptosis) and signaling pathways (ERK, AKT and survivin) as well as in organization of the cytoskeleton (Fig. 7.4b) (Masiello et al. 2014). When they cultured the microgravity-exposed cells under 1*g*, cells were enabled to recover their original phenotype (Masiello et al. 2014).

With the aim of identifying the underlying mechanisms of spheroid formation, Kopp et al. investigated the growth behavior of MCF-7 cells cultured on an RPM

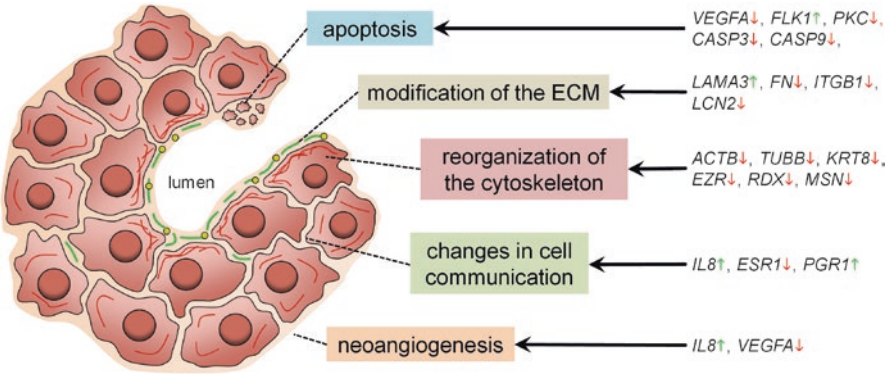

Fig. 7.5 Resembling of duct-like structures in a breast cancer MCS. Effects leading the MCS cells to resemble the ducts formed *in vivo* by human epithelial breast cells. Genes that enforce these effects and that were (compared to adherent cells) regulated after 5 days of clinorotation are listed on the *right*

in concert with changes in the expression of selected genes, playing a role in angiogenesis and tumor metastasis (Kopp et al. 2016). To focus on the short-term and long-term effects of s-µ*g* on breast cancer cells, they cultured the cells for 2 h, 4 h, 16 h, 24 h and 5 days, respectively, before they harvested adherently grown cells on the bottom of the culture dish and cells included in 3D aggregates separately (Fig. 7.4). One interesting finding was that the MCSs resembled the ducts formed *in vivo* by human epithelial breast cells (Kopp et al. 2016). To clarify this morphology, they measured the expression of 29 selected genes with a known involvement in MCS formation. Of those, *IL8*, *VEGFA* and *FLT1* were upregulated in 2 and 4 h adherent cell cultures. Regulated genes after 5 days of incubation are shown in Fig. 7.5. A pathway analysis revealed that the corresponding gene products are involved in the organization and regulation of the cell shape, in cell tip formation and membrane to membrane docking (Kopp et al. 2016). Of special interest is the downregulation of VEGFA in MCSs as high levels of circulating VEGF are a well-established indicator of poor prognosis for breast cancer patients (Jelkmann 2001).

Currently relevant anti-angiogenic agents in breast cancer therapy (i.e., bevacizumab, ramucirumab and sorafenib) also target the vascular endothelial growth factor system (Kristensen et al. 2014).

7.4.3 Effects of µg on the Extracellular Matrix

Various ECM components have been associated with poor prognosis in patients with breast cancer. Matrix proteins that are induced in breast cancer include fibrillar collagens, fibronectin, specific laminins and proteoglycans as well as matricellular proteins. Growing evidence suggests that many of these induced ECM

proteins play a major functional role in breast cancer progression and metastasis (Insua-Rodríguez and Oskarsson 2016). Also, cultivation of breast cancer cells on the RPM induces changes in the extracellular matrix. In the experiments by Kopp et al., RPM-exposure influenced mRNA expression of laminin, fibronectin, integrin-β1, ICAM1 and lipocalin-2 in MCF-7 cells (Kopp et al. 2016). In particular, fibronectin, which has attracted interest in cancer research (Fernandez-Garcia et al. 2014; Nam et al. 2010), owing to its role in tumor progression, is downregulated on the RPM. The upregulation of laminin-α3 could be involved in producing apical-basal polarity and the development of glandular structures within the MCS (Kopp et al. 2016).

7.4.4 Tumoroids and Histoids: Heterogeneous Breast Tumor Models

As breast tumors often contain diverse subpopulations of tumor cells with differing phenotypic properties, Vamvakidou et al. presented in 2007 an *in vitro* coculture-based three-dimensional heterogeneous breast tumor model out of the cell lines MDA-MB-231, MCF-7 and ZR-751 (Vamvakidou et al. 2007). These cells were co-cultured in an RWV and formed a large number of heterogeneous aggregates. The most important feature of these "tumoroids" is the temporal-spatial organization of solid tumors, including the presence of central necrotic areas and higher levels of cell division at the tumor periphery (Vamvakidou et al. 2007).

For mimicking tumor-fibroblast-interactions scientists have established 3D co-culture models of breast tumor cells and fibroblasts (Kunz-Schughart et al. 2001). Kaur and co-workers first studied the effects of microgravity and co-cultured breast cancer cells and fibroblasts in s-μ*g* for 9 days (Kaur et al. 2011). Co-cultures resulted in the generation of breast cancer "histoids" where cancer cells showed the invasion of fibroblast spheroids.

7.5 Studies on Skin Cancer: Malignant Melanoma

Melanoma is the most dangerous type of skin cancer. In 2012, it occurred in 232,000 people and resulted in 55,000 deaths worldwide (Stewart and Wild 2014). The incidence of this type of cancer is steadily increasing, but it is not clear to what extent changes in behavior, in the environment or in early detection are involved (Berwick and Wiggins 2006). The lack of appropriate experimental models for the study of this malignancy has hindered our understanding of cancer development and the rational design of effective therapies for a long time (Barth et al. 1995). With the aim of generating three-dimensional structures that mimic the *in vivo* tumor microenvironment much more closely than previous culture methods, Licato and co-workers carried out the first study with human melanoma cells using the effects of

microgravity (Licato et al. 2001). They cultured several primary human melanoma cells on an RWV for 7 or 8 days and observed spheroids. Immunohistochemical analyses showed multiple cellular types similar to the *in vivo* situation.

Later, Ivanova et al. investigated the morphology and cell behavior (cell division, attachment/detachment and migration) of highly metastatic BLM and low-metastatic 1F6 melanoma cells in s-μ*g* (Ivanova et al. 2011). Although human melanoma cells proliferate and migrate under s-μ*g* conditions without morphologic changes within the first 24 h, preliminary results suggested that the proliferation of the cells is reduced by simulated weightlessness. Further, they found that long-term exposure of human melanoma cells to s-μ*g* downregulates the mRNA levels of guanylyl cyclases A and B in comparison to 1*g* experiments. As the natriuretic peptide-sensitive guanylyl cyclase isoforms of guanylyl cyclase are expressed in cancer cells including melanomas, and natriuretic peptides have been implicated in cancers (Ivanova et al. 2001; Kong et al. 2008; Vesely 2005), their finding may indicate that the metastatic potential of melanoma cells could be attenuated at reduced gravity.

In a recent study, Zhao et al. showed that s-μ*g* promotes the apoptotic response of BL6–10 melanoma cells through a combined modulation of the Uev1A/TICAM/TRAF/NF-κB-regulated apoptosis and the p53/PCNA- and ATM/ATR-Chk1/2-controlled DNA damage response pathways (Zhao et al. 2016).

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Chapter 8
Outlook: Future Potential of Biotechnology Research in Space

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Abstract As has been shown in the previous chapters, biotechnology research in space has led to significant scientific breakthroughs and technological developments with great application potential. This is true for protein crystallization: here, significant progress in structure determination of certain molecules could be achieved with the help of improved crystals grown in space thereby supporting drug discovery and design. It is also true for certain aspects of cell biology; here, not only the basic understanding of gravity perception, transduction and response is in the focus of the scientists, but also the application of the results for tissue engineering and cancer research. The perspectives for the exploration era and for health research are discussed in some detail taking also into account that the utilization of the International Space Station ISS is secured until at least 2024.

Keywords Protein Crystallization • Cell Biology in Space • Space Exploration • Health Research

8.1 Perspectives for Protein Crystallization in Space

The method today most commonly used for protein crystallization experiments on ISS is the capillary based counter diffusion, based on the Granada Crystallization Box. The counter diffusion hardware is known to be most robust, fulfills all todays transport demands, compensates and tolerates to some extent unpredictable delay times and can be prepared to take advantage of long term crystallization experiments feasible on ISS. Presently a number of crystallization experiments are in preparation to be performed on ISS, which are coordinated by the space agencies ESA, DLR, JAXA and NASA. Most of these experiments are today performed and prepared by consortia of scientists, working together to optimize crystallization

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conditions and supporting each other in evaluating crystals obtained together as well. Besides conducting crystallization experiments of proteins and other biomolecules of high scientific interest and with potential applications in biotechnology or drug discovery, crystal growth diagnostics is again back in the focus of several ISS based protein crystallization experiments.

Considering that latest third generation synchrotron radiation sources with micro focus beam lines require today "only" much smaller crystals than mandatory only a few years ago, the demand for producing large crystals is no longer the only priority in microgravity crystallization experiments. Instead, the production of micro- and nano-sized crystals with excellent and superior quality is a new focus of today's microgravity crystallization experiments. This demand is accompanied and further pushed by the recently established revolutionary method to collect diffraction data termed serial femto second crystallography (SFX). SFX is today implemented at X-ray free electron lasers (XFELs) and several advanced third generation synchrotron radiation beam lines. This type of data collection requires instead of large single crystals a bulk amount (10^6–10^9) of micro- or nano-sized crystals.

As impurities, such as partially misfolded or partially denaturated proteins, or its aggregates, or other unwanted impurities occasionally present in crystallization solutions, reduce the final crstal quality, latest activities in microgravity crystallization experiments focus towards analyzing phenomena causing the incorporation of impurities in growing crystals, for comparison in microgravity and under lab conditions. Data obtained will allow to further optimize the production of macro- and micro-sized crystals and to avoid the incorporation of impurities as much as possible. To accomplish these goals, future microgravity crystallization experiments on the ISS plan also to analyze kinetics and pathways of the early processes of crystallization, like the formation of lipid dense clusters, the precursor of crystal nuclei, applying new and advanced analytical tools.

8.2 Perspectives for Cell Biology Research in Space

Research in cell biology remains a priority for several space agencies around the world. Especially for the ISS many new projects are currently under preparation—among them also three German experiments from the universities of Hohenheim and Magdeburg. One experiment will examine the adaptation of neuronal cells to weightlessness. The focus lies on the formation of sodium channels in neuronal cells in space compared to the formation on Earth. A second study will investigate the effect of real microgravity on primary human macrophages cells. The science team will study the influence of microgravity on the immune function including migration, presentation of antigens, cell to cell communication, and cell metabolism in macrophages. The third experiment will focus on the impact of real microgravity on human follicular thyroid cancer cells. This space experiment will clarify the underlying mechanisms of three-dimensional growth in space. The researchers will investigate changes in the proteome and secretome of the cells cultured in space on

the ISS. Furthermore, experiments studying three-dimensional growth behavior of cancer cells in space will be performed.

In addition, several German research groups funded by DLR Space Administration are preparing their experiments for TEXUS sounding rocket flights in the next years. Three teams will focus on microgravity-induced changes in the cytoskeleton of three different cell types via live cell imaging with the recently developed FLUMIAS device (fluorescence microscopy system for live-cell imaging in space). Further experiments on TEXUS missions and parabolic airplane flights with human cells will follow to investigate early gene expression changes in order to increase our knowledge on the influence of short-term microgravity on cells.

For the future, space exploration programs and commercialization of manned and unmanned spaceflight mission are in planning by space agencies around the world. There is a broad consensus among the agencies on having man-tended free flyers or space-station like platforms in low-Earth orbit also in the post ISS era for space-related research, technology demonstrations and preparation activities for space exploration missions. There are also manned exploration missions in preparation, not only by space agencies, but also by private companies like Space-X, which aim to bring humans to Moon or Mars. Such exploratory missions provide numerous new challenges and health related risks like radiation effects and risk of traumatic injuries and emergencies in space. A critical aspect here is the healing process including wound healing and suture behavior. Therefore, an international ISS project aiming to study the behavior and healing of wounds and sutures under microgravity conditions is currently under preparation.

Gravitational biology, cancer research, cell and molecular biology in space have demonstrated how cell exposure to microgravity influences various biological processes in different cell types. Thus, spaceflight provides unique conditions to study the underlying mechanisms. Proteome and secretome studies and following pathway analyses will detect altered proteins of human cells after microgravity-exposure. These proteins might be future drug targets in cancer research. Moreover, ground-based facilities will be important for the preparation of spaceflights and will serve as interesting machines providing profitable conditions for tissue-engineering purposes. Therefore, space research will contribute to the field of translational regenerative medicine. In addition, multicellular spheroids (MCS) developed in space and on ground-based microgravity-simulation devices can deliver important insights in tumor biology. These MCS are a model of micro metastases or avascular tumors and can be used for drug testing of anti-chemotherapeutic drugs or tyrosine kinase inhibitors and others. Summarizing these aspects and taking into account the commercialization of spaceflights, cell biological experiments in space are of great value and will become even more important in the near future for the pharmaceutical industry and for our health on Earth.